Effects of Persistent Herpesviral Infections on the Adaptive Immune System

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

von Thomas Felix Marandu

aus Dar es Salaam / Tansania

1. Referent:	Professor Dr. Michael Steinert
2. Referent:	Professor Dr. Ulrich Kalinke
eingereicht am:	29.05.2013
mündliche Prüfung (Disputation) am:	16.08.2013

Druckjahr 2013

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Publikationen

Cicin-Sain, L., Brien, J.D., Uhrlaub J.L., Drabig, A., Marandu, T.F. & Nikolich-Zugich, J. Cytomegalovirus infection impairs immune responses and accentuates T-cell pool changes observed in mice with aging. Plos Pathogens 8: e1002849 (2012)

Tagungsbeiträge

Marandu, T.F. & Cicin-Sain, L.: Herpesviral infections permanently alter the peripheral CD8 T-cell pool. (Poster) WS4-124. 2011 Joint annual meeting (Italian society of immunology, clinical immunology and allergology (SIICA) and German society for immunology (DGFI), Riccione (Italy) (2011)

Marandu, T.F., Finsterbusch, K., Kröger, A. & Cicin-Sain, L.: Effects of persistent herpesviral infections on the adaptive immune system. (Poster) 7.16. International herpesvirus workshop, Calgary (Canada) (2012).

Marandu, T.F., Finsterbusch, K., Kröger, A. & Cicin-Sain, L.: Effects of persistent herpesviral infections on the adaptive immune system. (talk) 7.16. International herpesvirus workshop, Calgary (Canada) (2012).

Marandu, T.F., Finsterbusch, K., Kröger, A. & Cicin-Sain, L.: Effects of persistent herpesviral infections on the adaptive immune system. (Poster) 7.16. HZI graduate school summer school, Rügen (Germany) (2012).

Acknowledgements

I would like to thank my supervisor Prof. Dr. Luka Čičin-Šain for his mentorship and for giving me the opportunity to work in his group. I do appreciate his mentorship which has made my knowledge in the immunology and virology field stronger. My sincere gratitude goes to my university mentor Prof. Dr. Michael Steinert and my thesis committee members Prof. Dr. Ulrich Kalinke and Dr. Siegfried Weiß for their advice and contributions of ideas on this study.

Thanks to my sponsor the Dar es Salaam University College of Education under the Science and Technology Higher Education Project for giving me the PhD scholarship, and the Helmholtz Gemeinschaft for funding the project.

This work would not have been possible without the support from my colleagues in the Immune aging and Chronic Infection (IMCI) group. Special thanks to Iryna Dekhtiarenko, Jennifer Oduro, Franziska Dag, Julia Holzki, Dr. Rosaely Casalegno, Dr. Adrien Weingärtner, Dr. Lisa Borkner, Inge Hollatz, Nils Hapke, Dr. Linda Ebermann, Ayse Barut and Jennifer Wolf for the technical support and advise; Ilona Paprotta and Anja Drabig for the technical support and introducing me to various lab facilities when I started working in the group. I would like to thank Dr. Andrea Kröger for her technical advice, Dr. Katja Finsterbusch for her help on the neutralization assay.

I would like to express my gratitude to the secretaries of the department of vaccinology Jenni Giesbrecht and Cornelia Senske also Angela Walter from the HZI international office for facilitating my travel and living in Germany.

A special thanks goes out to my friends in Germany especially my best friend Daniel Maeda for his advice and support before I came to Germany and during the whole period of my study.

Last but not least I would like to thank my parents Mr. and Mrs. Felix Marandu, my lovely sisters Sia, Naike, Gladness, Lightness and Diana for always praying for my career success.

Table of contents

List of figures

Abbreviations

ACK	Ammonium-Chloride-Potassium (K)
APC	Allophycocyanin
APCs	Antigen Presenting cells
ATCC	American Type Culture Collection
BCR	B Cell Receptor
BD	Becton, Dickinson and Company
BFA	Brefeldin A
CM	Central Memory
D2B6	DBA2 strain of a mouse crossed with C57BL/6 strain
EM	Effector Memory
FACS	Fluorescent Activated Cell Sorter
ffu	fluorescent focus forming units
FITC	Fluorescein Isothiocyanate
FoxP3	Forkhead box P3
HBSS	Hank's Balanced Salt Solution
HIV	Human Immunodeficiency Virus
HSV-1	Herpes Simplex Virus-1

HZI	Helmholtz-Zentrum für Infektionsforschung
i.n.	Intranasally
i.p.	Intraperitoneally
IE	Immediate Early
IFNγ/IFNg	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kb	Kilo base
MCMV	Murine Cytomegalovirus
MHV-68	Murine Herpesvirus-68
NK	Natural Killer
ORF	Open Reading Frame
p.i.	Post infection
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PE	Phycoerythrin
PerCp	Peridinin-chlorophyll-protein complex
PFU	Plaque Forming Unit
RPMI	Rosewell Park Memorial Institute

TCR	T Cell Receptor
TNFα	Tumor Necrosis Factor Alpha
VACV	Vaccinia Virus
VSV	Vesicular Stomatitis Virus

Abstract

Herpesviruses are divided into the α, β and γ subfamilies. More than 90% of the human population carries at least one, and more often a combination of several latent herpesviruses. Latency is the ability of a virus to silently persist in the host, and to reactivate upon episodes of immune suppression. During latency, the immune system is weakly, but persistently stimulated by viral antigens, and the effects of such burden on the homeostasis and function of the immune system remain unclear. To address this question, mice were infected with α, β or γ herpesviruses, individually or in combinations. There were no obvious changes in the homeostasis of the B cells, but an irreversible and very large increase of activated CD8 T cells, especially in mice carrying latent mouse cytomegalovirus (MCMV), a β herpesvirus. To investigate if this change led to functional impairments of the immune response to a new infection, the latently infected mice were challenged with vesicular stomatitis virus (VSV). At day 7 or 14 post challenge, the number of CD8 T cells responding to a VSV antigen were not altered, but due to the huge increase in the size of the CD8 compartment, the fraction of VSV-responding cells was reduced in the blood (but not in lymphatic organs) of mice carrying latent MCMV. Latent herpesviruses did not affect the VSV responses in the CD4 T cell compartment, but there was a delay in the Immunoglobulin (Ig) class-switch in MCMV infected mice. In conclusion, infection with persistent herpesviruses resulted in permanent changes in the homeostasis but not the function of the T cells and in delayed Ig class-switch in MCMV infected mice. Due to the pervasiveness of latent herpesvirus infections, these results may have important implications for public health, but also for the design of CMV-based vaccines.

Chapter 1

1.0 Introduction

1.1.1 Herpesviruses

The family *Herpesviridae* is, according to the International Committee on Taxonomy of Viruses (ICTV), divided into three sub-families, *Alphaherpesvirinae* (*Varicellovirus, Simplexvirus, Mardivirus* and *Iltovirus* genera), *Betaherpesvirinae* (*Cytomegalovirus*, *Roseolovirus* and *Muromegalovirus* genera), and *Gammaherpesvirinae* (*Rhadinovirus* and *Lymphocryptovirus* genera). All members of the family Herpesviridae have a spherical morphology and are made up of four major components, namely: the envelope, the tegument, the capsid and the core (1, 2) (Figure 1.1). Herpesviruses have linear double stranded DNA with genome size between 125 and 240 kilobase (kb) encased in an icosahedral-shaped nucleocapsid. All members of *Herpesviridae* replicates their genome in the host cell nucleus and express their genes in a specific order, where the immediate-early (IE) genes (encoding regulatory proteins and transcription factors) are expressed first, then early genes which encode proteins necessary for DNA replication, and lastly the late genes, which encode structural proteins (2). IE genes are resistant to cycloheximide (CHX) treatment (a protein synthesis inhibitor) in contrast to early genes which are sensitive to CHX, but are resistant to phosphonoacetic acid (PAA) treatment (a DNA polymerase inhibitor). Late genes are sensitive to both CHX and PAA, enabling selective distinction of the gene classes by western and northern blot analysis (3-6). Herpesviruses can infect mammals, reptiles, birds, amphibians and fish. In humans, primary infection may cause mild to severe disease upon which the viruses persist in the host for life in the latent phase.

Latency is defined as the maintenance of the viral genome in host tissues in the absence of infective viral particles, as detected by conventional virological assays. CMV reactivation from latency is one of the main causes of morbidity and mortality among immunocompromised hosts, for instance among bone marrow/solid organ transplant recipients or acquired immunodeficiency syndrome (AIDS) patients. Shedding of the viruses is done mostly through saliva, and while other routes of shedding are not as common, they are still possible, for instance through genital and other secretions. The vast majority of people carry herpesviruses in various combinations. Most people are positive for varicella, herpes simplex, cytomegalovirus and/or Epstein - Barr virus (7).

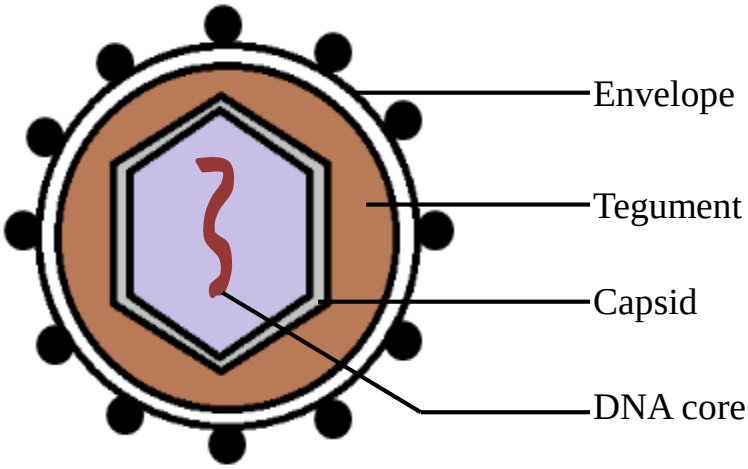

Figure 1.1: The four major components of herpesviruses

1.1.2 Latent infection with herpesviruses versus other chronic infections: Human immunodeficiency virus (HIV)

Both herpesviruses and HIV cause persistent infection and both may lead to disease progression once the host's immunity is compromised, but there are important differences. In HIV, the replication is active after the acute phase is cleared, and hence

the viral antigens constantly stimulate the immune system. Herpesviruses enter into a quiescent state after the acute phase with a low level of viral antigens exposed to the immune system (8) (**Figure 1.2**). HIV constantly stimulates the T cells, which may cause their exhaustion (9).This differs from the periodic stimulation of the immune system by latent herpesviruses, which may experience dormancy and reactivation cycles during latency (10). A long-term association of the herpesviruses and their natural host is speculated to have caused evolutionary adaptations in both the virus and the host, which favor a détente, where viruses do not harm the host, while not being attacked by the host's immune system.

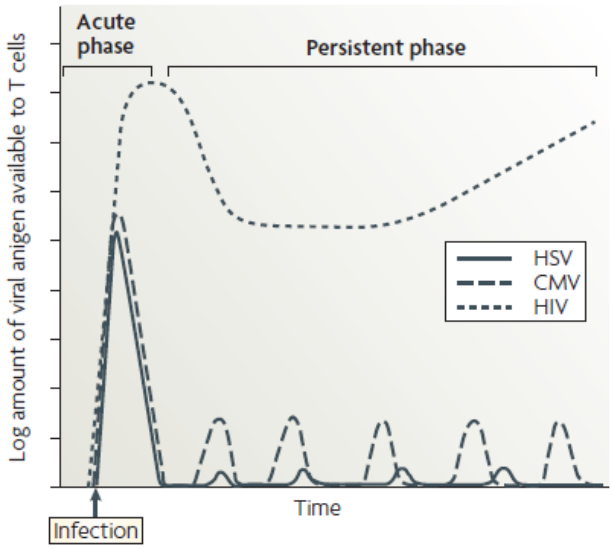

Figure 1.2: Persistent viruses and their antigenic load over time (adapted from (8))

1.2 Activation of the immune system by pathogens

The immune system consists of adaptive and innate immunity. Innate immunity is considered the first line of defense against pathogens and responds with the same magnitude upon a re-encounter of the same antigen, whereas adaptive immunity requires more time to respond, but has faster and more robust secondary responses when the same pathogen re-infects the host (11). The Adaptive immunity is mediated by two cellular compartments: the cell mediated immunity, which is mediated by αβ T lymphocytes and the humoral immunity which is based on the antibodies produced by plasma cells differentiated from B-cells. Cell mediated immunity deals predominantly with intracellular pathogens, whereas humoral immunity protects us against both intracellular and extracellular pathogens and their toxins. Furthermore, T cells are divided into helper (Th) and cytotoxic T lymphocytes (CTL), characterized by the surface expression of CD4 or CD8 receptors, respectively. Th lymphocytes, also referred to as CD4 T cells, exert their effects by helping either B cells or CTL (also known as CD8 T-cells), whereas CTL have a direct cytotoxic effect on their target. The cells that mediate innate immunity are natural killer cells and cells of the myeloid lineage including monocytes, granulocytes, macrophages, all phagocytic cells, eosinophils and basophils. Other cells of the immune system that appear not to fit clearly into either category, because they are at the intersection of the innate and adaptive immunity include gamma delta T (γδ T) cells and NKT cells (12, 13). In order to confer strong protective immunity against pathogens, the innate and the adaptive immune system work together but more details on how the innate immunity, γδ T cells, and NKT cells

are activated and respond to pathogens are not discussed here as it is beyond the scope of this study.

1.2.1 Activation of CD8 T cells

When viruses infect cells, some of the newly synthesized viral proteins are tagged by ubiquitin and degraded by proteasomes into small peptides, which are then transported into the endoplasmic reticulum and loaded onto the major histocompatibility complex-1 molecules (MHC I) expressed by all nucleated cells. The peptide-MHC I complexes are transported to the surface of the infected cell and presented to the CD8 T cells (14). Naïve T cells are activated in the secondary lymphoid organs. The migration of the naïve T cells into the lymph node is mediated by receptor molecules (CD62L and CCR7) expressed on their surface, which allow naïve cell homing to lymph nodes. Naïve CD8 T cells are activated by recognizing their cognate antigen (in this case a viral peptide-MHC I complex on the surface of the dendritic cells) with their TCR and by simultaneous binding of the co-stimulatory receptor (CD28) to their ligands on activated dendritic cells (CD80/CD86). The activation results in T-cell expansion and differentiation into effector cells (15). Effector cells are armed with cytotoxic granules (granzymes) and perforins which allow them to kill target cells. In addition, they can kill the target cell by the interaction of their FAS-ligand with the FAS receptor expressed on the surface of infected cells. Furthermore, effector cells can produce inflammatory cytokines such as TNFα and IFNγ, which have the potential to block viral replication either by killing infected cells or by repressing the viral gene expression. After activation, effector CD8 T cells loose CD62L and CCR7 from their surface which allows them to migrate out of the draining lymph node. Instead a new pattern of cell adhesion

molecules (CD11a, CD44) and chemokine receptors (CCR5, CXCR3) is expressed on their cell surface, which mediates their migration towards sites of inflammation and infection, where they may exert their cytotoxic effect on the virus-infected cells (16-18). Moreover, activated cells are characterized by the loss of expression of defined cytokine receptors at the surface such as the IL-7 receptor α chain (CD127), or the IL-15/IL-2 receptor β chain (CD122) (15, 19). Upon clearance of an infection, approximately 90% of effector T cells die by apoptosis leaving out about 10% of them which are then differentiated into memory cells which re-express the lymph node homing markers, but maintain the surface expression of adhesion markers that are typical of primed cells (CD11a, CD44) (15, 20).

1.2.2 Activation of CD4 T cells

CD4 T cells are classically divided into Th1 and Th2 T helper subsets. The non-classical subsets includes T-helper 17 (Th17), follicular helper T cells (Tfh) and regulatory T cells (Tregs). All the T helper subsets are defined by distinct cytokine secretion patterns. Th1 cells are typically involved in the host defense against intracellular pathogens such as viruses, whereas Th17 secrete cytokines (IL-17, IL-22) that recruit neutrophils to the site of infection and control extracellular pathogens such as bacteria and fungi. Th2 cells also function to defend the host against extracellular pathogens such as helminthes infection (21, 22). Tfh cells are found in the lymphoid tissues and they mediate humoral immune response by their interaction with the B cells (23, 24). Tregs suppress the immune function by secreting anti-inflammatory cytokines (IL10 and TGFβ) (25). Upon activation, Th1 cells can either directly kill the virus infected cells, by producing TNFα, which interacts with the TNF receptor p55 on the surface of infected cells and results in

caspase-dependent apoptosis, or they can produce Th1 cytokines (TNFα and IFNγ) which activate macrophages (26-29). CD4 T cells are activated by recognizing immunogenic peptides on the surface of professional antigen presenting cells (dendritic cells) in the context of the MHC II molecules. Peptides that are loaded on MHC II molecules are derived from proteins (host or viral), which are degraded in the endosomes by proteolytic enzymes, and therefore may be acquired by endocytosis from surrounding cells. As in the case of MHC I restricted peptides, the dendritic cells migrate to the lymph node to present MHC II peptides to naïve CD4 T cells, which are thus activated, essentially in the same manner as CD8 T cells (14, 30). CD4 T cells express surface molecules which closely match those on CD8 T cells, and enable them to migrate to and from the secondary lymphoid organ to the site of infection. In addition activated CD4 T cells up-regulate CD40L which in turn binds CD40 on the dendritic cells and up-regulates the MHC expression and the expression of co-stimulatory (CD80 and CD86) molecules on the surface (31-33). Activated CD4 T cells expand and differentiate into effector cells, whose numbers contract upon the clearance of the infection, mainly by apoptosis. A small fraction (about 10%) of effector cells typically remains and differentiates into memory cells.

1.2.3 Activation of B cells

B cells are produced in the bone marrow and then migrate to the secondary lymphoid organs. Activation of the B cells by antigens starts in the B cell zones of secondary lymphoid organs. During this process, naïve B cells through their B cell receptor (BCR) recognize the antigen, internalize, process and then load it onto MHC II molecules. These partially activated B cells migrate to the T and B cell zone border and interact

with follicular CD4 T cells. This process leads to an expansion of the B cells, formation of the germinal center as well as differentiation of B cells into memory cells and antibody secreting plasma cells (34, 35). Simultaneously, the immunoglobulin isotype switches. The constant region of the BCR changes from IgM and IgD to IgG, IgA or IgE (36). The activated and expanded B cells in the germinal center undergo a selection process in which the clones with low affinity BCRs for the antigen die by apoptosis, leaving predominantly high affinity clones, which can be recruited into the memory pool. Upon re-infection with the same pathogen, memory cells differentiate immediately into plasma cells that secrete high-affinity antibodies that are specific for the pathogen. Antibodies can neutralize toxins and free viruses, hence preventing their dissemination. Antibodies are also responsible for opsonization of pathogens, tagging them for phagocytosis and enzymatic digestion by macrophages. The ability of old individuals to mount a robust humoral immunity upon infection or vaccination is lower than in young ones, mainly due to the decline in the formation and function of the germinal center (37, 38). It is not clear whether adult hosts infected with single or multiple persistent herpesviruses have more pronounced deterioration in the function of the B cell compartment than their non-infected counterparts and if the effect is more pronounced with one type of herpesvirus than the others.

1.2.4 Immune aging

Immune aging (also called immunosenescence) is the loss of the immune function, coupled to an increase in inflammatory conditions which occurs in old age. Hallmarks of immunosenescence include the reduction of the number of newly generated naïve cell in the thymus caused by its involution, while the number of differentiated and terminally

differentiated T cells increases (39, 40). Immunosenescence should not be confused with the immune risk phenotype (IRP), where the latter is a set of alterations in the T cell compartment of elderly people which are predictive of significantly shortened life expectancy. Individuals with IRP have an inverted CD4/CD8 ratio, poor proliferative T-cell responses to polyclonal Ag stimulation and an increase in the absolute count of T cells with CD45RA+ CD28- CD27- CD57+ phenotype (41, 42).

Whereas the global improvement of socioeconomic conditions has improved the life expectancy of people, leading to a dramatic increase in the population of people who are aged 60 years and above, immunosenescence predisposes them to suffer or even die from emerging infections. Therefore, it is important to understand the factors that may accelerate or slow the onset of immunosenescence and suggest measures that may improve the lives of the elderly. Due to practical consideration, the majority of human studies on immunosenescence have relied on cross-sectional comparison of young and elderly adults. Longitudinal studies are more feasible in animal models, especially in animals which have a short life span, such as mice with a life span of up to three years. Experiments in primates, allow the acquisition of data from animal species which are closely related to humans and therefore bear highly relevant information, yet are constrained by the relative length of the life-span in most non-human primates.

A study in aged mice has associated the decline in CD8 T-cell immune responses to influenza to the decline in T cell repertoire (TCR) diversity in the naïve CD8 pool. Since thymectomized young mice had similar negative effects as aged mice, these data suggested that the involution of the thymus is a plausible cause of the decline in naïve T cell repertoire diversity (43). Experiments in rhesus monkeys infected with modified

vaccinia Ankara strain (MVA) also showed poor CD8 T-cell responses in aged monkeys, and an association to the loss of naïve CD8 T cells and constriction in the TCR repertoire diversity (44). Other studies have shown that recent thymic emigrants (RTEs) and CTL from aged mice respond poorly to stimulation and produce less IL2 as compared to those from the young mice suggesting that cell intrinsic defects of naïve T-cells may also contribute to immunosenescence (45, 46). The B cell compartment is also affected by the aging process, which is manifested as the decline in ability of follicular CD4 T cells in the germinal center to provide a cognate T-cell help and subsequent reduced ability of B cells to undergo class-switch recombination, the process which is crucial for an effective and robust humoral response (47).

Cytomegalovirus infection has been proposed as a contributor to immunosenescence. Studies in human and murine systems have shown that thymectomized, CMV infected young host have premature immune aging, when compared to CMV negative (48, 49). This suggests that persistent antigenic stimulation of hosts with no functional thymus and small populations of naïve cells (such as old individuals) may increase the susceptibility to new infections and reduce the T-cell response to vaccinations, as observed in the aged hosts (48, 50).

It remains unclear whether the effect of CMV infection on the ability of the immunosenescent host to respond to a new infection would be more pronounced in hosts primed with the persistent virus at a young age, or in those primed at a later time in the adult age. Moreover, it is not clear whether herpesviruses from other sub-families accelerate immunosenescence in the same way as it has been described in CMV infection.

1.3 Modulation of the adaptive immunity by α-herpesviruses: Herpes simplex virus type 1 (HSV-1)

Herpes simplex virus is a ubiquitous virus that naturally infects humans. The seroprevalence of HSV in the adult population is between 60-95% worldwide (51-53). Upon infection, the virus activates CD8 T cells that clear the primary infection and generate memory cells. HSV-1 establishes latency in the sensory ganglia and in the central nerve system (CNS). HSV-1 infection may cause fever and fever blisters during the acute phase and reactivation from latency. Reactivation of the virus may occur in immunocompromised individuals or can be induced when an individual is under physical or emotional stress during which the virus migrates from the site of latency into the periphery (54-56).

In vivo studies on alpha-herpesviruses are done on HSV-1 because the virus can readily infect mice as compared to other common alpha-herpesviruses infecting humans such as HSV-2 and varicella zoster. Studies in the mouse model have shown that systemic HSV-1 infection of C57BL/6 mice causes an accumulation of CD8 T cells specific for a peptide (SSIEFARL) derived from glycoprotein B (gB) with a kinetic that is characterized by a progressive accumulation of these cells from day 45 post-infection (57). On the other hand, local infection with HSV-1 (ocular infection) resulted in low-level maintenance of memory gB specific CD8 T cells after the contraction of the acute phase. Interestingly, the level of these cells were maintained at low level until 18 months post infection, but was then followed by a dramatic expansion, which resulted in cell counts of peptide specific cells which were higher than the initial peak value reached during the acute phase of infection (58). It remained unclear whether the expansion of

the gB specific CD8 T cells was due to weak control of the latent virus (perhaps caused by deterioration in the function of CD4 T cells or B cells) and reactive increase in the number of activated CD8 T cells in the old age. CD8 T cells specific for the HSV-1 gB peptide have a central memory phenotype in long term infected old mice (CD122+CD127+CD44+), which contrasts with MCMV specific CD8 T cells (discussed in detail in the next section) (58). Moreover, the expanded populations of HSV1 specific CD8 T cells that express KLRG1 maintain their ability to proliferate when transferred into RAG1 knockout mice (59). In a recent study, Smithey *et al.,* compared the effects of life-long MCMV and HSV-1 infection and found out that CD8 T cells responses to heterologous infection were comparable in both infections, but only mice infected with MCMV, alone or in combination with HSV-1, showed significantly impaired ability to control the novel infection (60).

HSV-1 specific CD4 T cells have shown to be important for clearing HSV-1 in neural tissue in a manner that is independent of Fas/perforin (60). Another study has shown that CD4 T cells help the gB specific CD8 T cells to control HSV-1, and that in absence of CD4 T cells the HSV-1 specific CD8 T cells expressed the inhibitory receptor PD-1 (61). Since most of the studies in HSV-1 infection were focused on the CD8 T cell compartment, it remains unclear if the virus affects also CD4 and/or B cells. Finally, most of these experimental models have focused on HSV-1 infection in isolation, in absence of other herpesviral infections, although HSV-1 infection usually occurs in people that are infected with multiple herpesviruses. Therefore, the experimental infection with multiple herpes viruses may also offer insights to understand how herpesviruses may affect the adaptive immune system.

1.4 Modulation of the adaptive immunity by β-herpesviruses: Cytomegalovirus (CMV)

Cytomegalovirus is a ubiquitous virus carried by 50% – 80% of the population in developed and 90 – 100% of people in developing countries (62, 63). Primary infection with the virus can cause fever or can also be asymptomatic. Congenital infection with CMV may cause birth defects such as hearing loss, blindness and learning disabilities in newborns. Moreover, reactivation of the virus from latency in immunocompromised hosts such as stem cell or organ transplant recipients on immunosuppressive therapy and AIDS patients may cause retinitis, pneumonitis, colitis, encephalitis and graft rejection (64, 65). CMV is species specific, but viruses that coevolved with different species have very similar genomes and interact with similar facets of the immune system of their hosts. This allows the study of CMV pathogenesis and interaction with the host in animal models.

Longitudinal studies in cohorts of aged people that focused on the effects of latent CMV on T cell immunity have shown an association between CMV seropositivity and phenotype changes in the T cell compartment (66-68). Infection with CMV has been proposed as one of the main environmental factor accelerating immunosenescence and changing the immune system towards IRP (68-73). Findings in the mouse model of CMV infection supported this hypothesis. Monitoring of MCMV specific CD8 T cells over a long period of time showed that there is an expansion of selected immunodominant epitopes, a phenomenon known as “memory inflation” (74-76). The inflated CD8 T cells keep the reactivation episodes of the virus in check and their accumulation suggests that, each reactivation episode may lead to recruitment of new

naïve T cells into the memory pool (77). It was shown that by replacing the IE1 anchoring residue leucine with the alanine residue on the MCMV genome resulted into IE3 transcription and higher IE1 transcripts during latency, which suggested CD8 T cells were constantly suppressing the reactivation events by sensing the IE1 protein presented by MHCI molecules on the infected cells (78)

We have recently published that mice infected with MCMV, but not with vaccinia exhibited a reduced frequency of CD8 T cells responding to a challenge with unrelated viruses (e.g. West Nile or flu virus). Furthermore, we have shown that the fraction of CD8 T cells responding to the challenge inversely correlates to the fraction of effector memory CD8 T cells (Figure 1.3). However, in this study we did not address the question whether the absolute numbers of these cells was also reduced.

We proposed that the increase of the frequency of memory T cells with the late differentiated phenotype and the decrease of the frequency of naïve T cells in CMV infected hosts might impair the immunity to the challenge pathogen either because they lower the number of naïve T cells available for responding to novel infections, or (2) lead to a competition for immunological space.

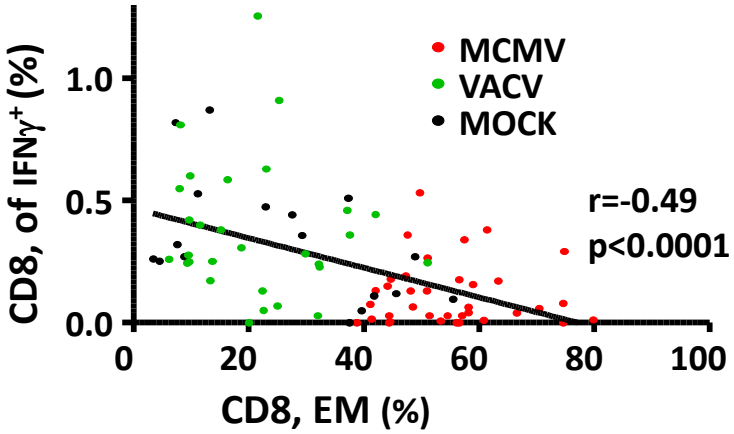

Figure 1.3: Correlation between frequencies of CD8 T cells responding to West Nile virus and EM CD8 T cells in adult mice long-term infected with MCMV, VACV or nothing (mock). (Adapted from (79))

Studies on CD4 T cell responses to human CMV have shown a negative correlation between the number of viral DNA copies and the number of CD4 T cells specific for the virus (80). Moreover, these cells showed a late differentiated phenotype (CD27-CD28- and short telomere), suggesting the involvement of CD4 T cells in the control of the virus (81). In the mouse model of CMV infection, CD4 T cells were shown to contribute to the inflation of CD8 T cells (82, 83). Monitoring of the kinetic of MCMV specific CD4 T cells by restimulation with a library of overlapping CMV peptides revealed no long-term accumulation of CMV specific CD4 T cells, but rather the maintenance of a stable number of CD4 T cells after clearance of the primary infection (84). There are no available studies on the effect of latent CMV infection (alone or in combination with other infections) on the homeostasis and function of the peripheral CD4 pool. Results from Trzonkowski *et al.,* suggested CMV infection was associated with elevated proinflammatory cytokines (TNFα, IL6, IL-1β) and low level of serum anti-hemagglutinin titers after influenza vaccination (85). However, these results were not supported by

another study from a Dutch cohort of elderly people in long-term care facilities where they found no association between CMV infection and poor responsiveness to influenza vaccination (86). Experiments in the mouse model showed that B cells are not necessary for the control of primary virus infection, but that MCMV disseminates much more efficiently upon reactivation in B cell deficient mice suggesting that the B cells play an important role in blocking virus dissemination upon reactivation (87). However, the effect of persistent herpesvirus infection on the phenotype of B cells and the quality of antibodies produced against new infections has not been studied.

1.5 Modulation of the adaptive immunity by γ-herpesviruses: Epstein - Barr virus (EBV) / Murine herpesvirus-68 (MHV-68)

Approximately 90% of adult people are estimated to carry EBV asymptomatically (88, 89). Primary infection usually occurs during childhood through saliva contact and may be asymptomatic or, in some cases, may cause fever and lymphadenopathy (90, 91). Upon clearance of the primary infection, the virus establishes life-long latency in B cells. Reactivation from latency can occur in severely immunocompromised individuals. Moreover, EBV infection may cause Burkitt's lymphoma, Hodgkin's lymphoma or nasopharyngeal carcinoma (92) and therefore the virus was categorized as a 'carcinogenic agent' by the world health organization in 1997 (93). Studies to understand pathogenesis and immunity against gamma herpesviruses utilize the established prototypic Murine gammaherpesvirus clone 68 (MHV-68, γHV68 or Murid herpesvirus-4), which is closely related to the Kaposi sarcoma-associated herpesvirus and EBV (94-96). The virus was isolated from the bank voles (*Clethrionomys glareolus*), its natural host (97), but it is well-able to infect laboratory mice (94, 98) and establish

latency not only in the B cells as EBV, but also in the dendritic cells and macrophages (99, 100).

CD8 T cells are important to control the acute phase of MHV-68 infection and to suppress reactivation of the virus from latency. Two well-characterized MHV-68 immunodominant epitopes (ORF6 and ORF61) are commonly used to study CD8 T cell responses against MHV-68 in C57BL/6 mice. On the other hand, no inflationary MHV-68 peptide has been identified so far, which differs from MCMV or HSV-1 infected (101). In humans carrying EBV, most of the EBV-specific CD8 T cells show a central memory phenotype and no sign of memory inflation except an accumulation of these cells in tonsils, at sites of latent infection (102).

Studies on the function of CD4 T cells during MHV-68 infection have shown that these cells can suppress the virus replication independently of CD8 T cells and B cells (103, 104). The suppression from reactivation and killing of virus-infected cells by CD4 T cells is mediated by IFNγ and direct cytotoxicity (105). It remains unclear, if the number of MHV-68 specific CD4 T cells increases over time. Similarly, the phenotypes of MHV-68 specific memory CD4 T cells have not been described. Due to the fact that most people are EBV seropositive, it is difficult to determine if the presence of gamma-herpesviruses in the host may alter the functional capacity of the adaptive immune system to respond to emerging infections.

1.6 Detection of phenotypic changes and functional capacity of the adaptive immunity

Several markers on the lymphocyte subsets which are up-regulated or down-regulated upon infection have been identified by multi-parametric flow-cytometry of cells stained

with monoclonal antibodies against these markers. Furthermore, functional immunological assays allow the measuring of the size and the quality of the immune responses to antigens including, but not limited to, ELISPOT, ELISA *in vitro* and *in vivo* neutralization assays. *In vitro* antigenic restimulation followed by flow cytomety for intracellular cytokines or proliferation markers, allow the study of the immune phenotype and function.

T cells that are specific for a certain epitope may also be detected in flow cytometry or FACS-sorted by peptide-MHC multimers coupled to fluorochromes (106, 107). Commonly used multimers include tetramers, pentamers and dextramers (108, 109), where the main difference between multimers is the type of their backbone, and hence the number of MHC-peptide monomers and fluorochromes they carry. Tetramers have a streptavidin backbone and contain four MHC-peptide complexes and one fluorochrome bound to it while pentamers are composed of a pentameric complex backbone that binds five MHC-peptides and five fluorochromes. Dextramers are composed of a dextran polymer backbone that carries large number of MHC-peptide complexes which increases the binding avidity of the complex to the surface of a T-cell, and thus enhances the detection of low avidity antigen specific T cells recognizing low avidity targets.

The function of plasma cells derived from B cells is usually determined by the amount and quality of antibodies that are produced by them. While the ELISA assay is used to determine the physical concentration of antigen-specific antibodies in the serum, the *in vitro* neutralization assay allows the testing of their functional capacity (usually expressed as serum dilution that is still sufficient to neutralize the target virus.

In this study, the phenotype and the function of the adaptive immune system of mice infected with various herpesviruses were analyzed by a combination of multicolor flow-cytometric analysis and *in vitro* neutralization assay.

Chapter 2

2.0 Aim of the thesis

Several recent *in vivo* studies showed that herpesviruses have the potential to irreversibly affect the adaptive immune system (49, 79, 110). The studies were focused on the CD8 T cell subset and identified life-long phenotypic changes in hosts carrying herpesviruses, like CMV or HSV-1. However, these studies focused on infections with one single herpesvirus in isolation and only on the T-cell compartment. While most people carry more than one type of herpesviruses, no study has compared the effects of single latent infection versus multiple infections with diverse herpesviruses in an experimental model, and no study addressed the effect of latent infections on cellular compartments beyond the CD8 T cells subset.

The aim of this thesis was to determine, in a mouse model of infection, whether latent herpesvirus infections affect the adaptive immune system of adult hosts and if they alter immune responses to an unrelated viral infection.

2.1 Specific objectives

1. To dynamically monitor the long-term homeostatic changes in the CD8 T cell compartment of mice carrying latent viruses in isolation, or in combination.
2. To determine the effects of the duration of the latent infection with α, β or γ herpesviruses (alone or in combination) on the ability of the adaptive immune system to respond to a new, unrelated infection.
3. To compare the effects of latent herpesvirus infections on the CD4 T cell or B cell response to a new, unrelated infection.

Chapter 3

3.0 Materials and Methods

3.1 Materials

3.1.1 Antibodies

Antibody/Multimer	Clone	Supplier
CD11a-PE-Cy7	2D7	BD, USA
CD127-PE	A7R34	Biolegend, USA
CD25-APC	PC61.5	eBioscience, Germany
CD28	R1-2	eBioscience, Germany
CD3-APC-eFluor780	17A2	eBioscience, Germany
CD44-Alexa fluor700	IM7	Biolegend, USA
CD44-FITC	IM7	Biolegend, USA
CD49d	37.51	eBioscience, Germany
CD4-APC	GK1.5	Biolegend, USA
CD4-FITC	RM4-5	eBioscience, Germany
CD4-Pacific Blue	GK1.5	Biolegend, USA
CD62L-eFluoro 605NC	MEL-14	eBioscience, Germany
CD62L-FITC	MEL-14	Biolegend, USA
FoxP3-PE	FJK-16s	eBioscience, Germany

IFNγ-APC	XMG1.2	Biolegend, USA
KLRG1-Biotin	2F1/KLRG1	eBioscience, Germany
NKG2A/C/E-FITC	20d5	eBioscience, Germany
Nkp46-PE	29A1.4	Biolegend, USA
Streptavidin-BV570	N/A	Biolegend, USA
TNFα-FITC	MP6-XT22	Biolegend, USA
VSV-dextramer-PE	N/A	Immudex, Denmark
αCD8-PerCp-Cy5.5	53-6.5	Biolegend, USA

3.1.2 Cell lines

Cell line	ATCC number	Supplier
BHK 21	CRL-13001	ATCC, USA
M2-10B4	CRL-1972	ATCC, USA
NIH3T3	CRL-1658	ATCC, USA
Vero	CCL-81	ATCC, USA

3.1.3 Chemicals

Chemical/Reagent	Supplier
2-Mercaptoethanol	Sigma-Aldrich Chemie, Germany
Brefeldin A	Sigma-Aldrich Chemie, Germany
Crystal violet	Serva, Germany
Ethanol	Fluka, Switzerland
Fetal calf serum	Greiner Bio-One, USA
Formaldehyde	Carl Roth, Germany
Heparin	Ratiopharm, Germany
Isofluran	Essex Tierarznei, Germany
Ketamine	CP-Pharma, Germany
L-Glutamine	Gibco, UK
Methylcellulose	Sigma-Aldrich Chemie, Germany
Penicillin/Streptomycin	Gibco, UK
Sodium Chloride	J.T Baker, The Netherlands
Xylazine	WDT, Germany

3.1.4 Buffers

Buffer/Media	Supplements	Company
ACK	1mM EDTA, 10mM $KHCO_3$, 155mM NH_4Cl, pH 7.3	
DMEM	N/A	Gibco, UK
FACS	1xPBS, 5% FBS	
Fixation buffer	N/A	eBioscience
Fixation/permeabilization buffer	N/A	eBioscience
HBSS	N/A	Appli Chem, Germany
PBS	2.7 mM KCl, 1.8 mM KH_2PO_4, 137 mM NaCl, 10 mM Na_2HPO_4, pH7.4	
Permeabilization buffer	N/A	eBioscience
RPMI 1640	N/A	Gibco, UK

3.1.5 Peptides

Peptide	Haplotype specificity	Sequence	Reference	Supplier
HSV-1 gB	H-2k^{b}	SSIEFARL	(57, 111, 112)	HZI, Germany
MCMV IE1 (pp89)	H-2L^{d}	YPHFMPTNL	(76, 113)	HZI, Germany
MCMV M139	H-2k^{b}	TVYGFCLL	(74, 114)	HZI, Germany
MCMV M38	H-2k^{b}	SSPPMFRV	(74)	HZI, Germany
VSV	H-2k^{b}	RGYVYQGL	(115)	HZI, Germany

3.1.6 Consumables & machines

Name	Supplier
1.4ml U bottom FACS tubes	Micronic, The Netherlands
1.5ml tubes	SARSTEDT, Germany
100µm Cell strainers	BD Falcon, USA
2ml tubes	SARSTEDT, Germany
50ml Falcon tubes	Cellstar Tubes, USA

Accuri cytometer	Accuri cytometers Inc. USA
Flat-bottom 96 well plates	Thermoscientific, Germany
Haemocytometer	BRAND, Germany
LSRII cytometer	BD, USA
Sonicator (Sonorex TK30)	Bandelin, Germany
Top table centrifuge	Thermoscientific, Germany
U-bottom 96 well plates	BRANDplates, Germany

3.1.7 Mice

Male DBA/2xC57BL/6 (D2B6) F1 mice were purchased from Janvier (Le Genest, France), 129S2/SvPasCrlxBALB/cAnNCrl F1 (129B) mice were purchased from Charles River (Sulzfeld, Germany). Mice were housed in specific pathogen free (SPF) conditions at the HZI animal facility. Animal experiments were done according to animal welfare guidelines by FELASA and GV-SOLAS and were approved by the Lower Saxony State Office of Consumer Protection and Food safety (permit number 33.9-42502-04-11/0426).

3.1.8 Viruses

In all the experiments we used a BAC derived molecular clone of the mouse cytomegalovirus (116) as our wild type virus (MCMV). The virus stock was made on M2-10B4 cells. The MHV-68 was kindly provided by Dr. H. Adler (117), Helmholtz Zentrum München, and the virus stock was produced on NIH3T3 cells. HSV-1 strain 17, VACV and recombinant VACV expressing an immunodominant peptide from HSV-1 (rVACV)

were obtained from Dr. J. Nikolich-Zugich (58, 118-120), University of Arizona, Tucson and grown on Vero cells. The Influenza virus PR8M which is closely related to the Mount Sinai strain of A/PR/8/34 (H1N1) referred to as “low pathogenic” virus was obtained from Dr. K. Schughart (121), HZI and was propagated in the chorio-allantoic cavity of ten days old embryonated chicken eggs for 48h at 37^0C. The vesicular stomatitis virus (VSV) Indiana strain, grown on BHK 21 cells, was given by Dr. Andrea Kröger (122), HZI.

3.2 Methods

3.2.1 Infection

a. Infection of the D2B6 mice

Six or two months old D2B6 mice were infected with herpesviruses, allowed to age till 15 or 17 months of age respectively and then challenged with VSV. Mice were divided into three independent cohorts each with six groups of mice i.e MCMV, MHV-68, HSV-1, VACV/rVACV, mock and the group which received a combination of the three herpesviruses at an interval of two weeks between infections (triple infection group). All the groups were infected intraperitoneally (i.p.) with a total volume of 200µl of the following doses of the viruses; MCMV $2x10^5$pfu, MHV-68 $1x10^6$pfu, HSV-1 $1x10^6$pfu, VACV $1x10^6$pfu. Nine and 15 months post priming with the herpesviruses, VACV or PBS, all the mice were challenged with $1x10^7$ pfu of VSV by intranasal (i.n.) application of 20µl of virus suspension after the mice were fully anesthetized by i.p. injection of a cocktail of ketamine/xylazine.

b. Infection of the 129B mice

129B mice were infected at the age of 6, 12 or 18 months with herpesviruses. At each time point two cohorts of mice (males and females) were infected either intraperitoneally with MCMV ($2x10^5$pfu), MHV-68 (10^6pfu), rVACV (10^6pfu), mock infected (200µl of PBS) or intranasally with Flu ($2x10^4$ffu). One group of mice received a combination of the two herpesviruses (MHV-68 and MCMV) at two weeks space between the first infection (MHV-68) and the next (MCMV). There was no HSV-1 group, because this mouse strain is highly sensitive to this virus. All the mice were challenged with 10^7 pfu VSV i.n. approximately at the same age (17-18 months).

3.2.2 Organs and peripheral blood collection/processing

Two blood specimens were acquired from the retrobulbar venous plexus of each mouse anesthetized by isofluran. The blood was diluted in 300µl of heparin in HBSS (2U/ml). The first specimen consisted of exactly 100µl and was used to determine absolute blood cell counts in an Accuri cytometer, while the second one had a volume of approximately 140µl and was used for *in vitro* stimulation assays. Erythrocytes were removed from blood by osmotic shock lysis of cell pellets in 9ml of water, followed immediately by the addition of 3ml of 4xPBS, upon which the cells were centrifuged and the pellet was transferred into a U- bottom 96 well plates for staining or *in vitro* stimulation. Mice were euthanized by CO_2 asphyxia and lymph nodes (mediastinal and inguinal) and spleen were harvested into 2ml tubes containing 1ml of RPMI. Spleen and lymph nodes were homogenized on 100µm-pore-size cell strainers, in 5ml of RPMI and erythrocytes were removed from spleen samples by ACK buffer lysis for 5 minutes. Spleenocytes were re-

suspended in 1ml RPMI, counted using a haemocytometer (Neubauer chamber) and a volume equivalent to approximately 500K cells was taken for *in vitro* stimulation and flow cytometric analysis. Lymph node homogenates were centrifuged and the pellet was re-suspended in 200μl of FACS buffer, 50μl was transferred into 96 wells plate for the cell counting in an Accuri cytometer, while the rest was used for the *in vitro* stimulation assay.

3.2.3 Preparation of the VSV lysate for the stimulation of CD4 T cells

VSV lysate was made by infecting BHK21 cells at 100% confluency with the VSV at MOI of 0.01 for 24 hours, resulting in throughout infection. The infected cells were harvested into 50ml Falcon tubes, centrifuged at 1000rpm, washed once with 2ml of PBS, and re-suspended in 3ml of PBS followed by three freeze-thaw cycles and sonication for 60 seconds to release viral proteins from the cells. This was followed by another centrifugation step (1200rpm for 5 minutes) and the supernatant containing viral proteins was harvested, aliquoted and stored at -20^0C until the stimulation assay. The mock lysate (uninfected BHK21 cells) was treated in parallel with the VSV lysate and used in control assays, to identify unspecific responses directed against BHK-21 proteins.

3.2.4 In vitro neutralization assay (IgM and IgG concentration)

Serum from the mice collected at day 7 or day 14 post VSV challenge was diluted 40-folds in minimal essential medium containing 5% of heat-inactivated fetal calf serum, which was followed by serial 1:2 dilutions in the same medium. The diluted serum was mixed with equal volumes of medium containing 500 pfu of VSV, incubated at 37^0C, 5%

CO_2 for 90 minutes, and transferred onto monolayers of Vero cells in 96-wells plates. Upon 60 minutes incubation at 37^0C, 5% CO_2, the monolayers were overlaid with DMEM containing 1% methylcellulose and incubated at 37^0C, 5% CO_2. 48h later, the overlay was removed and cells were fixed and stained in 0.5% crystal violet dissolved in 5% formaldehyde, 50% ethanol and 4.25% NaCl. After the plates dried, the serum dilution that reduced the number of plaques by 50% was taken as the titer. To determine the IgG titer, the serum was pre-treated with an equal volume of 280 mM 2-Mercaptoethanol for one hour at room temperature prior to dilution and sample processing as described above

3.2.5 Peptide and lysate stimulation of CD8 and CD4 T cells

All the peptides used in this study were synthesized at the HZI peptide facility and HPLC purified to reach 65-95% purity (for a list of peptide sequences see section 3.1.5). Lymphocytes were re-stimulated at 37^0C with peptides listed in section 3.1.5 diluted in RPMI-supplemented medium (5% fetal bovine serum, 1% Glutamine, 1% Penicillin/streptomycin) at a concentration of 1µg/ml for one hour followed by five additional hours of stimulation in the presence of 10µg/ml Brefeldin A (BFA). Co-stimulatory antibodies CD49d and CD28 were added in the RPMI/peptide mixture. For MCMV and the triple infection group, an L(d) restricted peptide derived from the IE1 protein was used and for the HSV-1 group, we used the K (b)-restricted peptide from the glycoprotein B of HSV-1 (HSV-1 gB) (see section 3.1.5). Samples from the mice after the VSV challenge were restimulated with a peptide derived from the VSV nucleocapsid protein N52-59 (123). Medium containing only the co-stimulatory antibodies (CD28 and CD49d) was used as a negative control for the stimulation. For

CD4 T cells stimulation, the VSV lysate or mock infected BHK21 cell lysate diluted in RPMI-supplemented medium and in the presence of the co-stimulatory antibodies was used to re-stimulate the cells for one hour followed by another eight hours stimulation in the presence of Brefeldin A. Stimulation of the lymphocytes was performed in U-bottom 96-well plates in a total volume of 100µl.

3.2.6 Immunofluorescence staining and flow cytometric analysis

Surface phenotypic markers on cells were stained prior to cell fixation and intracellular cytokine staining. Surface staining was performed with anti- CD62L-eFluor 605NC, αCD8-PerCp-Cy5.5, CD11a-PE-Cy7, KLRG1-Biotin, CD4-Pacific blue, CD44-Alexa Fluor 700, CD3-APC-eFluor 780, CD127-PE, Nkp46-PE, NKG2A/C/E-FITC and for intracellular staining the following antibodies were used: IFNγ-APC and TNFα-FITC. Biotinylated antibodies were detected by secondary staining with streptavidin-Brilliant Violet 570. Briefly, cells were incubated with the antibodies against the surface markers in a total volume of 50µl for 30 minutes at 4^{o}C in darkness followed by two washing steps with 200µl of FACS buffer. When biotinylated antibodies were used in the panel, an additional 15 minutes incubation step with streptavidin-brilliant Violet 570 was done, followed by two washing steps as above. Samples were suspended into 100µl of FACS buffer prior to acquisition in an LSRII apparatus or intracellular staining.

Intracellular cytokine staining was done as follows: samples were fixed in 100µl of fixation buffer for 5 minutes, followed by addition of 100µl of permeabilization buffer and incubation for 3 minutes. Cells were washed in permeabilization buffer and incubated with the intracellular antibodies diluted in permeabilization buffer for 30 minutes followed by two washing steps before the acquisition using the LSRII machine.

Samples for the absolute cell count were first incubated with the VSV-Dextramer-PE for 15 minutes in the darkness at room temperature which was followed by a 20-minute staining with the surface antibodies: CD4-APC, CD8-PerCP-Cy5.5, CD62L-FITC or CD44-FITC. Samples were washed twice with FACS buffer and then acquired.

Intracellular (nuclear) staining for regulatory T cells (Tregs) was done with the mouse regulatory T cell staining kit from the eBioscience company. Briefly, cells were surface stained for 30 minutes at 4^{o}C in the darkness with CD8-PerCp-Cy5.5, CD4-FITC, CD25-APC in a total volume of 50µl. Cells were then washed twice with 200µl of FACS buffer and incubated with 100µl of Fixation/Permeabilization buffer for 30 minutes in the darkness, which was followed by two washing steps with the permeabilization buffer. The cells were incubated with the Foxp3-PE antibody (clone FJK-16s; eBioscience) or with the isotype control in permeabilization buffer for 30 minutes at 4^{o}C in the darkness. Cells were then washed twice with the same buffer, suspended in 100µl FACS buffer and acquired in the Accuri cytometer.

3.2.7 Data analysis

FACSDiva software was used for acquisition of flow cytometry samples (BD, San Jose, USA), whereas Flowjo software (Treestar, Ashland, USA) was used for the analysis. Samples acquired by the Accuri cytometer were analyzed with CFlow software, version 1.0.227.4 (Accuri cytometers,Inc., Michigan, USA). Statistical analysis was done using Graphpad prism, version 5.04 (GraphPad software, Inc., California, USA).

Chapter 4

4.0 Results

4.1 HOMEOSTATIC CHANGES IN THE ADAPTIVE IMMUNE SYSTEM OF MICE INFECTED WITH HERPESVIRUSES

4.1.1 Infection with herpesviruses causes permanent changes in the CD8 compartment of mice

Three cohorts of D2B6 (F1) mice were infected with various herpesviruses, vaccinia (VACV) or mock infected at the age of 6 months and the phenotype of lymphocytes was monitored for a period of nine months (Figure 4.1). VACV was used as a paradigmatic non-persistent virus, to exclude that phenomena observed in herpesvirus infection were common to any infection. The fraction of CD8 effector memory (EM) cells in mice infected with HSV-1, MCMV, MHV-68 or a combination of the three herpesviruses was compared to the fraction of the same cells in mice infected with VACV, or to cells from the mock group (Figure 4.2). All infections resulted in an initial expansion of the CD8 EM cells, upon which the response contracted in VACV or HSV-1 infected mice to levels from the mock-infected group, while mice infected with MCMV, MHV-68 or a combination of the three herpesviruses showed almost no contraction of the EM pool, because its fraction was maintained at ~50-60%. Interestingly, the EM contraction in HSV-1 infection was transient, because by 13 weeks post infection their number started to gradually rise again and reached the same level as in mice infected with other herpesviruses (Figure 4.2B). The triple infection had small, but consistent, additive effects over the groups infected with a single Herpesvirus.

The response kinetic to the IE1 (pp89)-derived peptide YPHFMPTNL, which is an MHC-I restricted peptide associated with memory inflation in mice with H-2L^d haplotype was compared between mice carrying MCMV or a combination of the three herpesviruses. Mock infected mice were used as negative control (Figure 4.2C). MCMV-only infection induced a kinetic characterized by an expansion of the fraction of responding CD8 T cells from 2% until it reached 3.4% at week 21.7 and then stabilized, which was somewhat lower than the results previously observed in Balbc mice (75, 79). This discrepancy might be explained by the strains of mice used, as here D2B6 F1 mice were used (a cross of H-2K^d and H-2K^b haplotype) which increase the ability of cells from these mice to present peptides to the T cells and this might have led to peptide competition (124). Interestingly, response kinetic to IE1 in the triple infection group was characterized by an initial response peak at one week post infection followed by a sharp contraction until week four, upon which the fraction of peptide specific cells stabilized at approximately 1.4%.

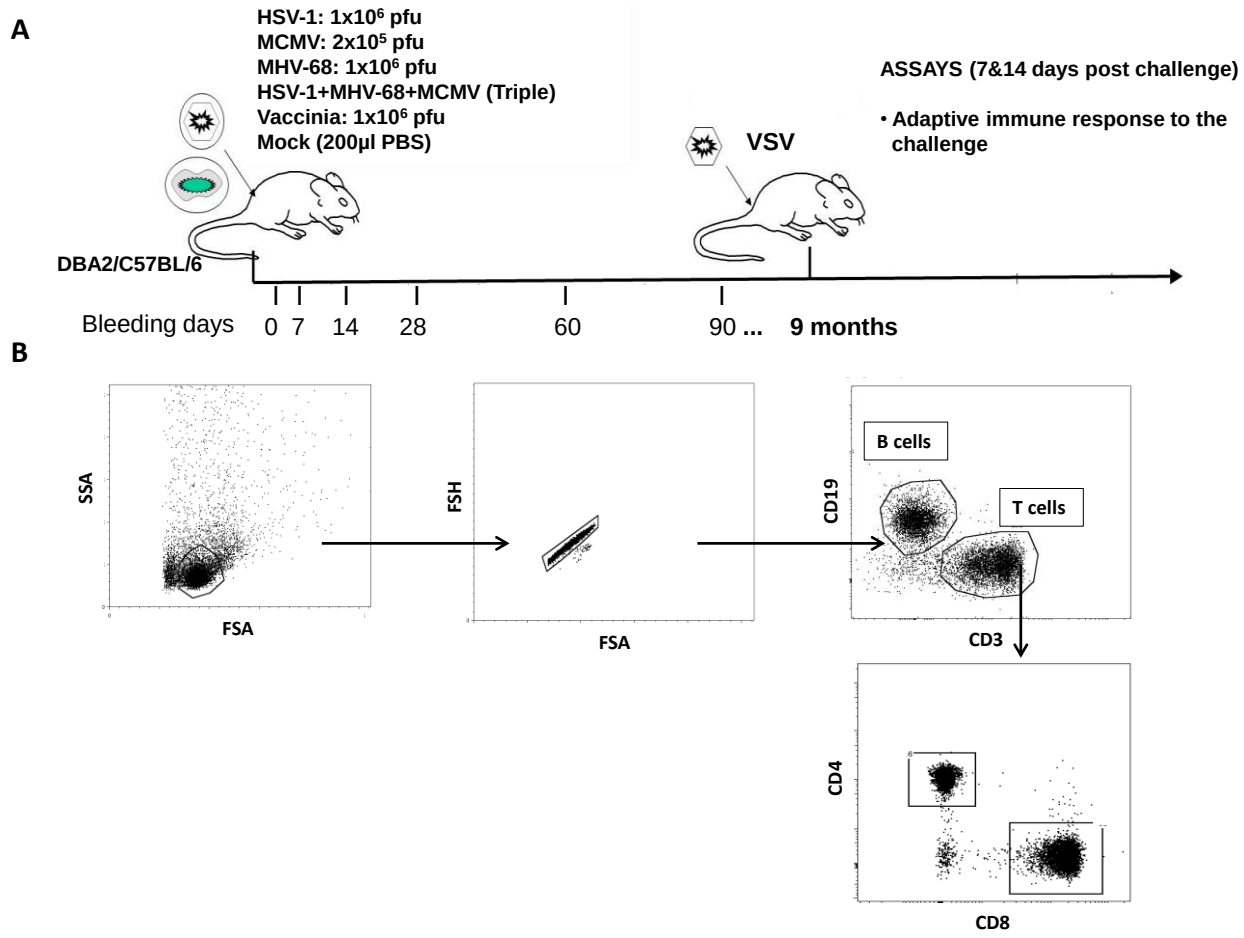

Figure 4.1: Experimental set up and gating strategies. (A) Three cohorts, each with six groups of D2B6 F1 mice were infected with the indicated virus (es) or mock infected (PBS) at the age of six months. Each group had at least 15 mice. The mice were bled at indicated time points to monitor changes in the phenotypes of T or B cells until nine months post infection (15 months of age), when all the groups were challenged with VSV. At day 7 and 14 post challenge, the T cells and antibody response to the VSV challenge were analyzed. (B) Progressive gating strategies used to identify the population of T and B cells are shown.

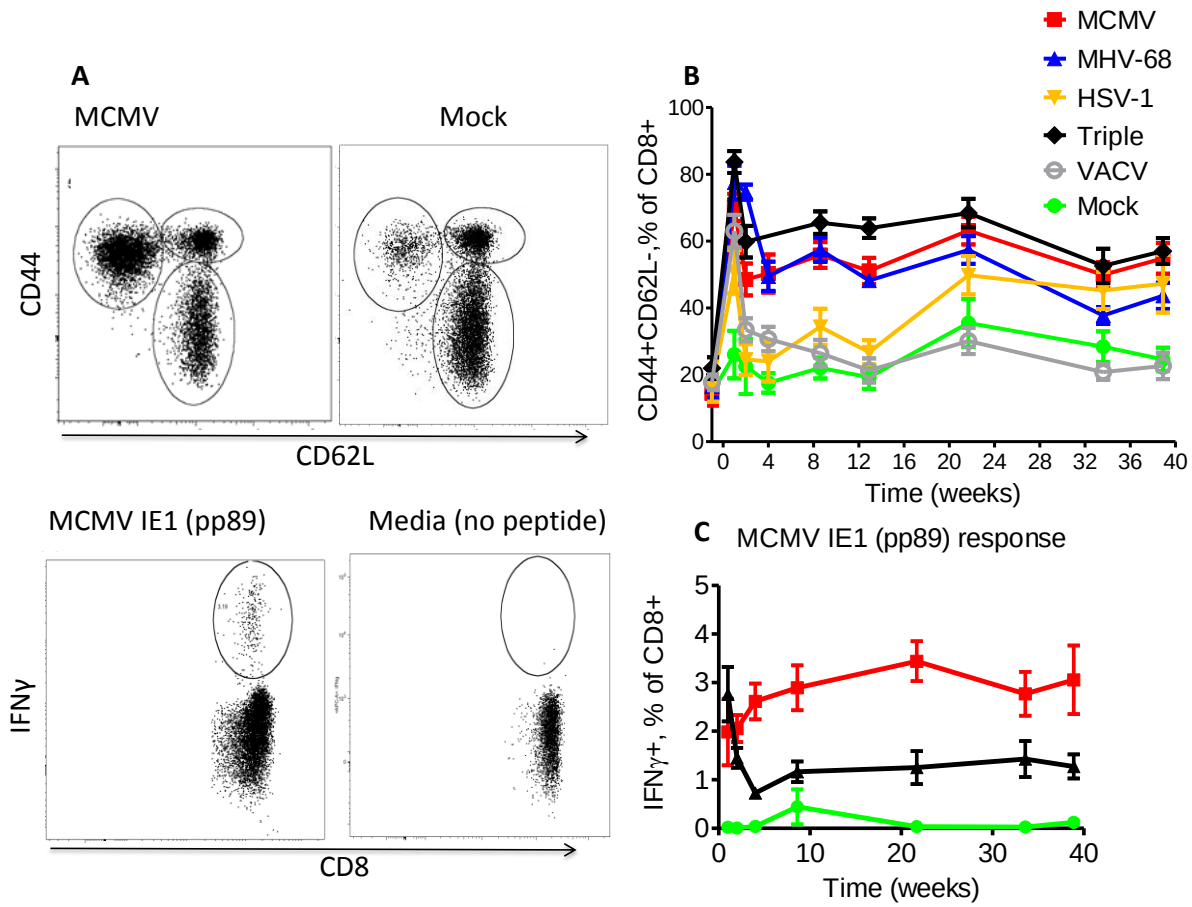

Figure 4.2: Herpesvirus infection results in permanent homeostatic changes in the CD8 Compartment. (A) Blood was collected before and after infection and CD8 T cell subsets were analyzed by multicolor flow cytometry to define the expression of CD62L and CD44 surface markers (upper panels) and intracellular IFNγ levels upon MCMV IE1 (pp89) peptide in vitro restimulation (lower panels). (B) Time kinetic of the fraction of CD8 EM from infection until nine months later, (C) Peripheral blood leukocytes from the MCMV, mock or triple infected group were collected over a period of nine months. Lymphocytes were stimulated *in vitro* with the MCMV IE1 (pp89) peptide and the kinetic of CD8 T cells responses, assessed by IFNγ intracellular staining is shown.

The frequency of responding CD8 T cells were compared to other MCMV peptides with the inflationary phenotype (M38 and M139) between the MCMV group and the triple infection group using another D2B6 cohort of mice infected for one year and there was

a similar trend towards lower fractions of these cells in the triple infection group (Figure 4.3A). On the other hand, the fraction of CD8 T cells responding to the HSV-1 immunodominant peptide from single infection with HSV-1 was comparable to the triple infection group (Figure 4.3B), arguing that the weaker response to MCMV peptides was a phenomenon associated with MCMV infection only. The discrepancy between the MCMV-infection and the triple-infection group in the frequency of CD8 T cells specific for the MCMV inflationary peptides might be due to a competition for antigen-specific CD8 cells in multiple infections, in line with the observation that the total EM pool was only mildly increased. Alternatively, since infection of the mice was done in an order that mice were first infected by HSV-1 then MHV-68 and lastly MCMV at two weeks gap between one infection to the next, MHC-I down regulation as a viral immune evasion strategy of the HSV-1 and/or MHV-68 might have played a role. Further studies are needed to ascertain the validity of these hypotheses.

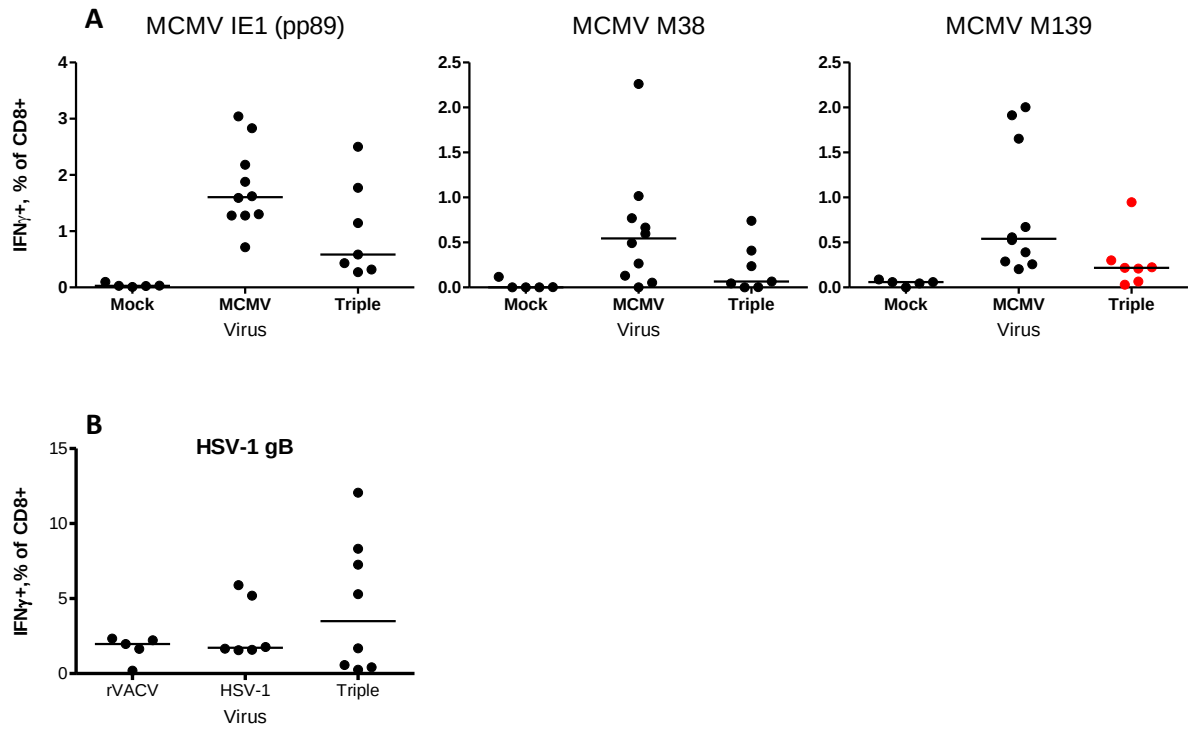

Figure 4.3: MCMV and not HSV-1 response to the inflationary peptides is reduced in mice carrying multiple herpesviruses. (A) D2B6 mice infected with $2x10^5$ pfu MCMV, a combination of the three herpesviruses (10^6pfu HSV-1, 10^6pfu MHV-68 and $2x10^5$pfu MCMV) or mock-infected at the age of two months were bled after 14 months. The blood leukocytes were split into three parts and *in vitro* stimulated with three MCMV inflationary peptides, followed by intracellular staining for IFNγ response. (B) D2B6 mice infected as above were bled and the leukocytes were stimulated with the HSV-1 gB peptide and stained for IFNγ. Each dot represents a mouse and the horizontal line represents median of the group.

4.1.2 Infection with MCMV but not with other herpesviruses significantly increase the frequency of CD8 T cells expressing KLRG1 and NKG2A/C/E

Natural killer and memory CD8 T cells express a C-type lectin receptor (KLRG1) and the CD94/NKG2 heterodimer. The expression of KLRG1 on cytotoxic T cells from virus infected mice has been described as a phenotype which is associated with proliferative

senescence (59, 125). CD94/NKG2 heterodimers interacts with non-classical MHCI molecules (HLA-E in humans and Qa-1^b in mice). The three isoforms of NKG2 which are well studied are NKG2A, NKG2C and NKG2E where NKG2A provides an inhibitory signal and the other two an activation signal. Studies have shown that, NKG2A up-regulation on CD8 T cells during virus infection and the interaction with the Qa-1^b molecules on the APCs inhibits MHC I restricted cytotoxicity (126, 127). Moreover, expression of KLRG1 and NKG2A on CD8 T cells from MHV-68 infected mice has been shown to confer more protection to a challenge with the same virus (128). Several studies on CD8 T cells expressing NKG2A use a monoclonal antibody which detects all the three NKG2 isoforms (NKG2A/C/E) with the reason that NKG2A is the dominant transcript expressed by nearly all antigen-specific CD8 T cells and on the other hand the expression of NKG2A and NKG2C are mutually exclusive (129-131). In this study, the phenotypes of NK and CD8 T cells were analyzed (Figure 4.4), to determine whether there is a difference in the fraction of CD8 T cells expressing KLRG1 or NKG2A/C/E in herpesvirus-infected and the mock group. Their fractions were compared nine months post infection (Figure 4.5 upper panel), and to control if changes were specific for the CD8 fraction, or a reflection of general upregulation of KLRG1 and NKG2A, their expression on NK cells were also compared (Figure 4.5 lower panel). The fraction of CD8 T cells expressing KLRG1 was significantly higher in the MCMV infected and in the triple-infected group than in the mock-infected controls. HSV-1 and MHV-68 infection showed a trend towards increased responses, but it did not reach statistical significance (Figure 4.5 upper panel). The same results were observed in the fraction of

CD8 T cells expressing NKG2/A/C/E (Figure 4.6 upper panel). On the other hand, there were no significant differences in the fraction of NK cells (Figure 4.6 lower panel).

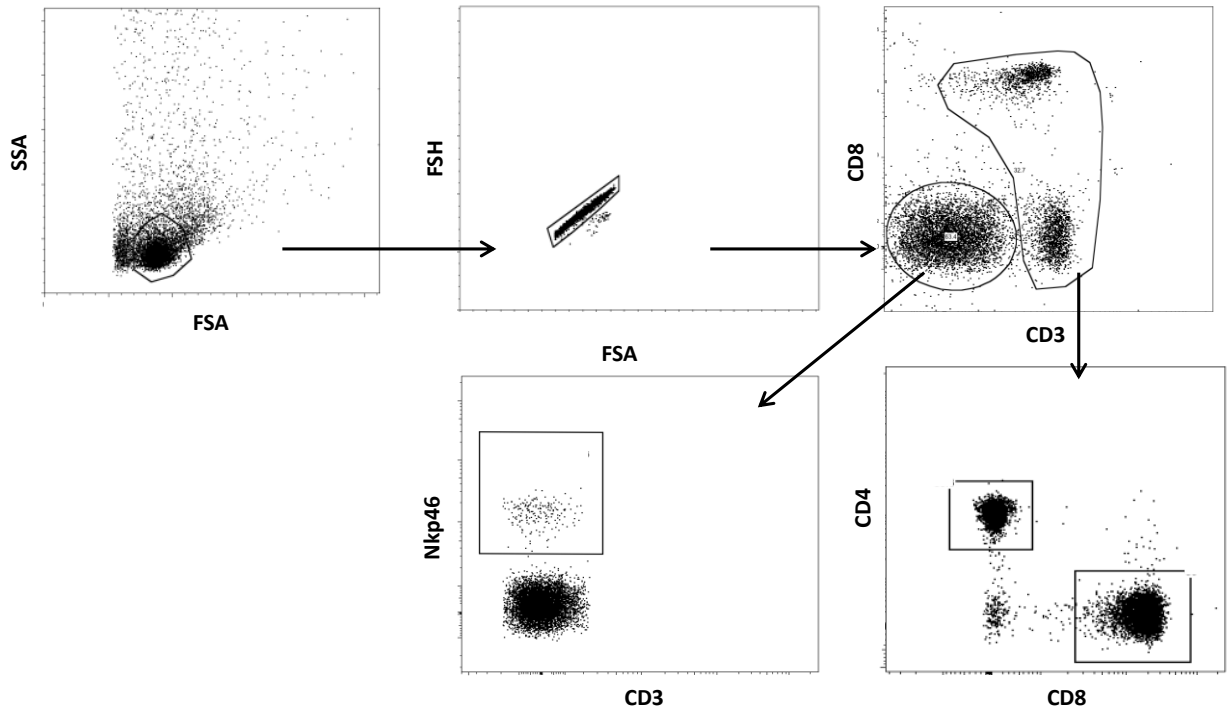

Figure 4.4: Progressive gating strategies used to identify the population of T and NK cells. Nine months post infection, PBMCs from the D2B6 mice were surface stained to compare changes in the phenotypes of the CD8 T cells and NK cells. Cells gated as NKp46+CD3- or CD4-CD8+ were analyzed in details on panels in figures 4.5 and 4.6.

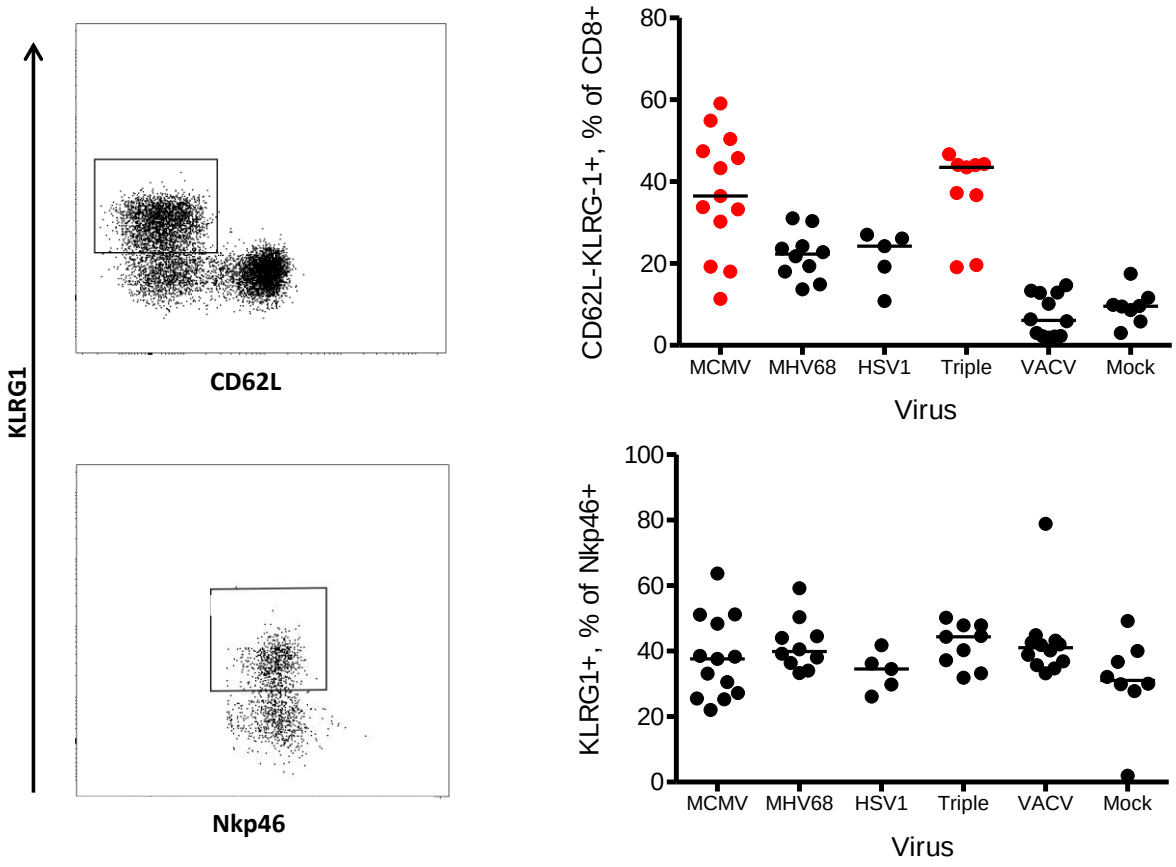

Figure 4.5: Infection with MCMV significantly expands the fraction of CD8 EM cells expressing KLRG1. Nine months post infection, the fraction of $CD62L^{-}KLRG1+$ CD8 T cells (top panel) and KLRG1+ NK cells (bottom panel) in infected mice was compared to the mock infected control group. Each dot represents a mouse, the line represents the median and the red dots shows groups which had $p < 0.05$ (Kruskal-Wallis test followed by Dunns post-analysis).

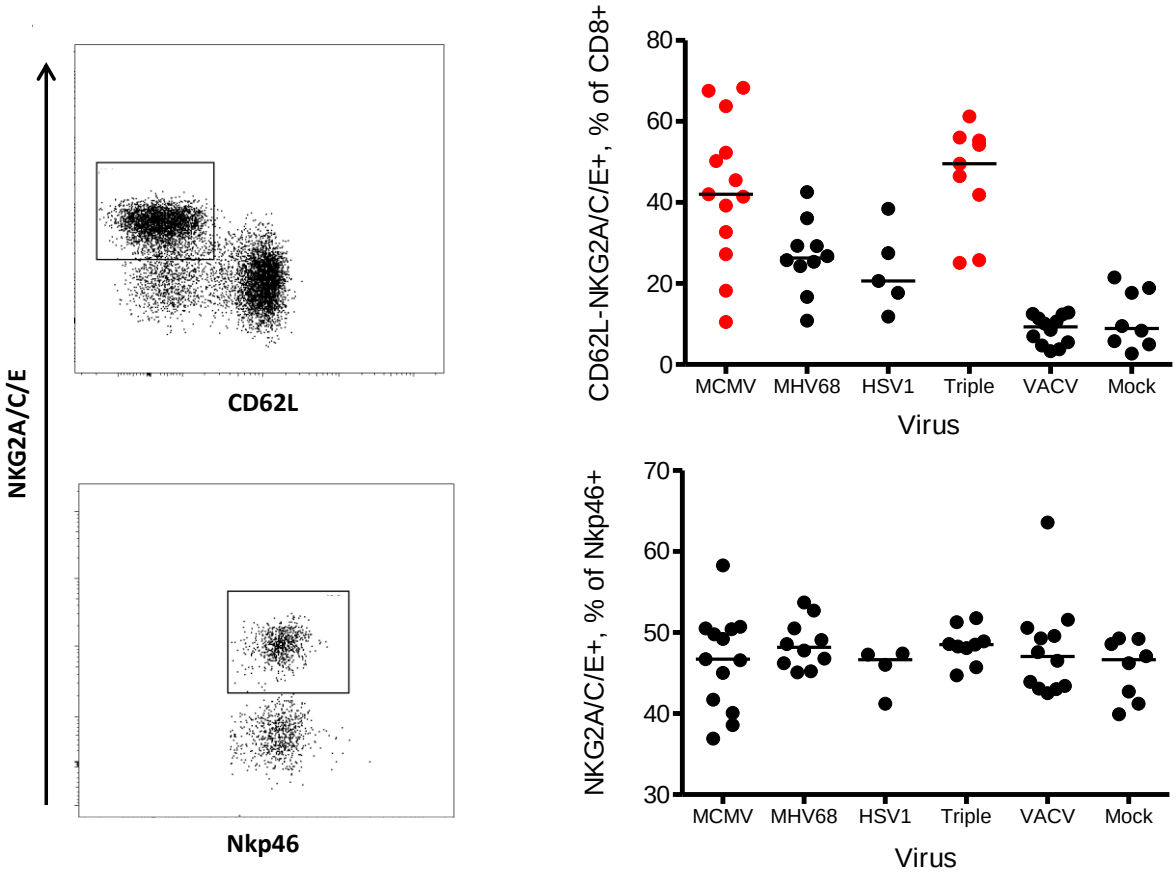

Figure 4.6: Infection with MCMV significantly expands the fraction of CD8 EM cells expressing NKG2A/C/E. Nine months post infection, the fraction of CD62L$^-$ NKG2A/C/E+ CD8 T cells (top panel) and NKG2A/C/E+ NK cells (bottom panel) in infected mice was compared to the mock infected control group. Each dot represents a mouse, the line represents the median and the red dots shows groups which had $p<0.05$ (Kruskal-Wallis test followed by Dunns post-analysis).

4.1.3 Infection with multiple herpesviruses result to an expanded frequency of CD4 EM cells

Eight months post infection; the fraction of CD4 T cells displaying EM phenotypes in infected mice was compared with the mock-infected control group (Figure 4.7). The

triple-infected group showed a statistically significant increase in the fraction of CD4 EM cells, whereas mice infected with single herpesviruses showed a similar trend but did not reach the level of statistical significance. The VACV group showed no obvious difference in the frequency of these cells.

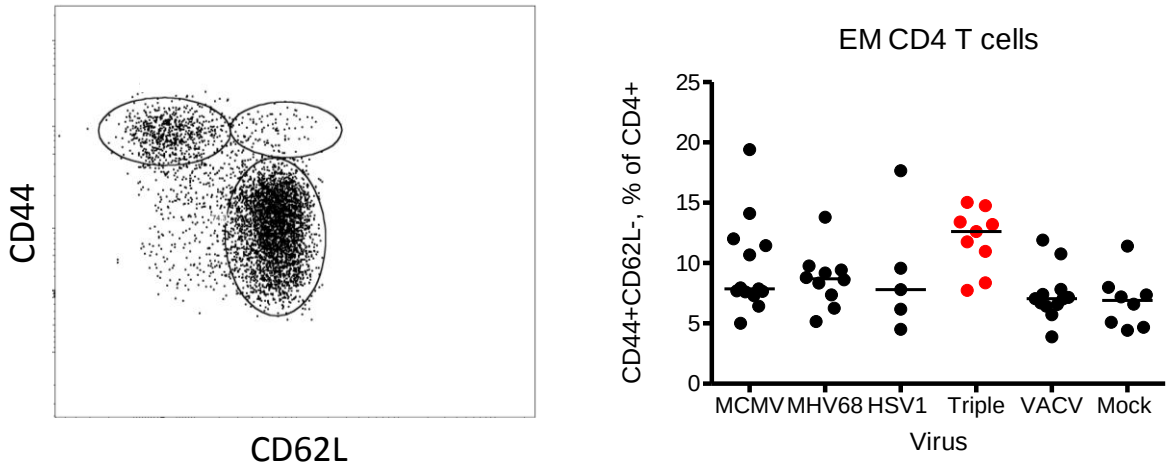

Figure 4.7: Multiple herpesvirus infection expands the fraction of CD4 T cells with EM phenotype. Eight months post infection of D2B6 mice, the CD4 T cells from the peripheral blood were analyzed for the expression of CD44 and CD62L. The frequency of CD62L-CD44+ cells was compared by Kruskal-Wallis test followed by Dunns post analysis. Each dot represents a mouse and the line is the median of the group. The group with $p < 0.05$ is indicated by red dots.

4.1.4 Infection with MHV-68 only result into decreases in the frequency of regulatory T cells but not the absolute count

Regulatory T cells (Tregs) are known to secrete suppressive cytokines (IL10 and TGFβ) and thus suppress the immune response of effector T-cells. I*n vitro* studies with human peripheral blood mononuclear cells (PBMC) have shown that Tregs may suppress the anti-viral responses against human CMV and HIV (132). Elevated frequencies of CMV-

specific EM CD8 T cells have been also shown in IL10 knock-out mice infected with MCMV, and since these mice had lower copy numbers of latent MCMV genomes than the wild type mice, this argues for enhanced control of viral replication by pro-inflammatory cytokines (133). To determine if latent herpesvirus infection altered the frequency and the absolute number of the Tregs, the fraction and the absolute number of CD4 T cells expressing the transcription factor Foxp3 and the surface receptor CD25 were analyzed at one-year post infection (Figure 4.8A). Only the MHV-68 group showed a significantly reduced frequency of Tregs as compared to the mock-infected group, whereas other herpesviruses showed a similar trend, but no statistically significant difference (Figure 4.8B upper panel). On the other hand, the absolute number of Tregs in the mice infected with the herpesviruses was not significantly different in any group (Figure 4.8B lower panel). Interestingly, infection with vaccinia virus resulted in relative and absolute Treg counts that were slightly increased, but not to a point of statistical significance.

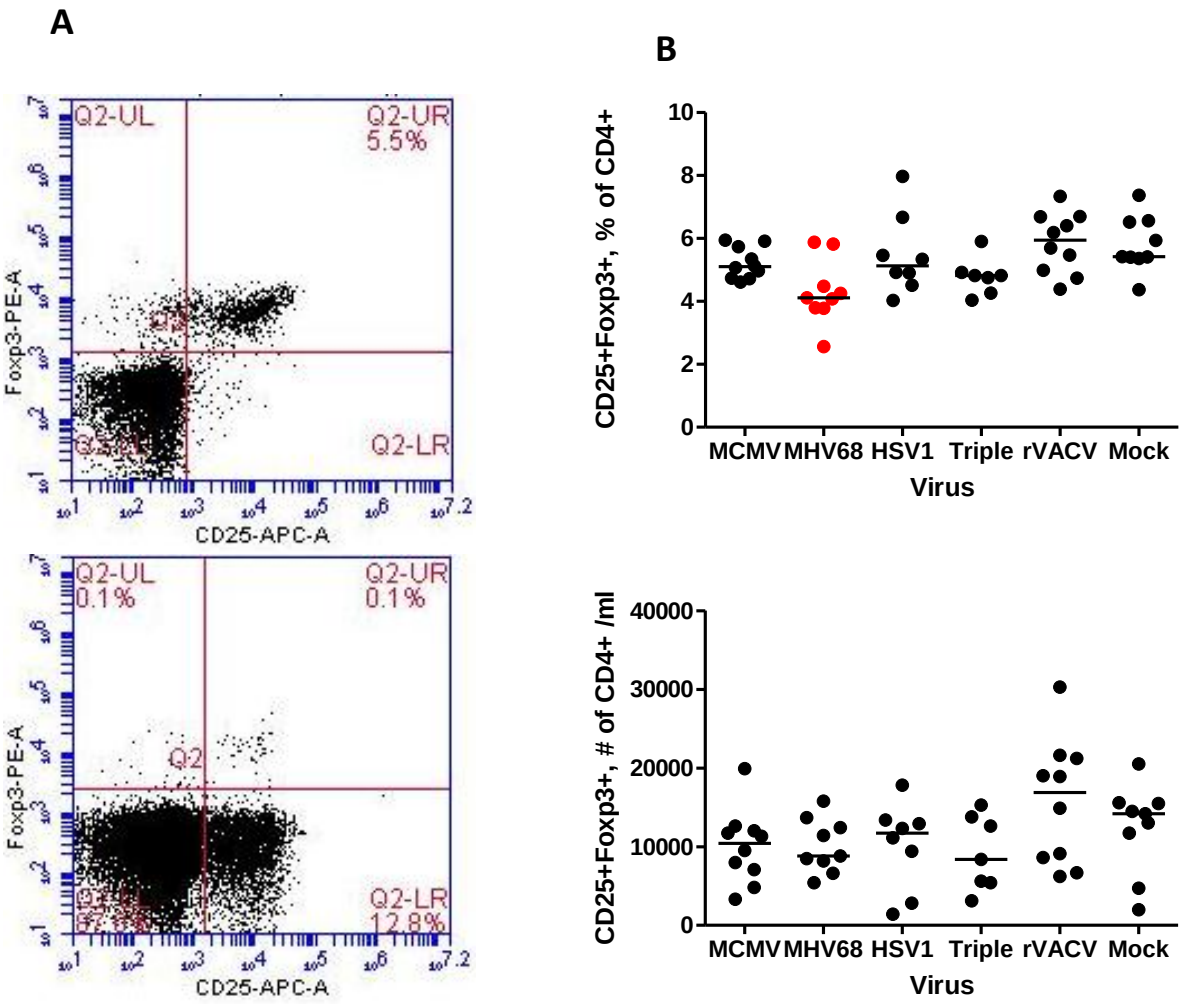

Figure 4.8: The fraction of Tregs but not the absolute count is reduced in the MHV-68 infected mice. (A) Cells from the CD4+ gate were analyzed on the expression of CD25 and Foxp3 (upper plot). The lower dot plot show cells stained with the isotype-control antibody for FoxP3 (rat IgG2a), to exclude unspecific antibody binding. (B) D2B6 mice infected with the indicated viruses were bled at one year post infection and PBMCs were stained for Tregs markers (CD4+, Foxp3+ and CD25+). The upper panel shows the percentage of Tregs in the CD4 pool and the lower panel shows their absolute count per ml of blood. Each dot represents a mouse and the horizontal line represents median of the group. The group with red dots had $p<0.05$ (Kruskal-Wallis test followed by Dunns post analysis

4.1.5 Herpesviruses do not affect the peripheral memory B cells compartment

There are two kinds of memory B cells which are distinguished by the expression of IgM (134-136) ; conventional memory B cells which do not express IgM but IgG and IgM memory B cells which express IgM but not IgG. The latter is said to persist longer after the clearance of an infection whereas IgG+ memory cells are described as the frontline responders to infections (137). Eight months post infection, the blood lymphocytes were stained for memory B cell markers. The fraction of both “conventional” memory B cells and IgM memory B cells were analyzed on expression of CD3- CD19+ CD80+IgM- and CD3-CD19+CD80+IgM+ respectively (Figure 4.9). The fraction of memory B cells was comparable in all the infection groups and for both types of memory subset, suggesting that long-term herpesvirus infection does not affect this subset of lymphocytes.

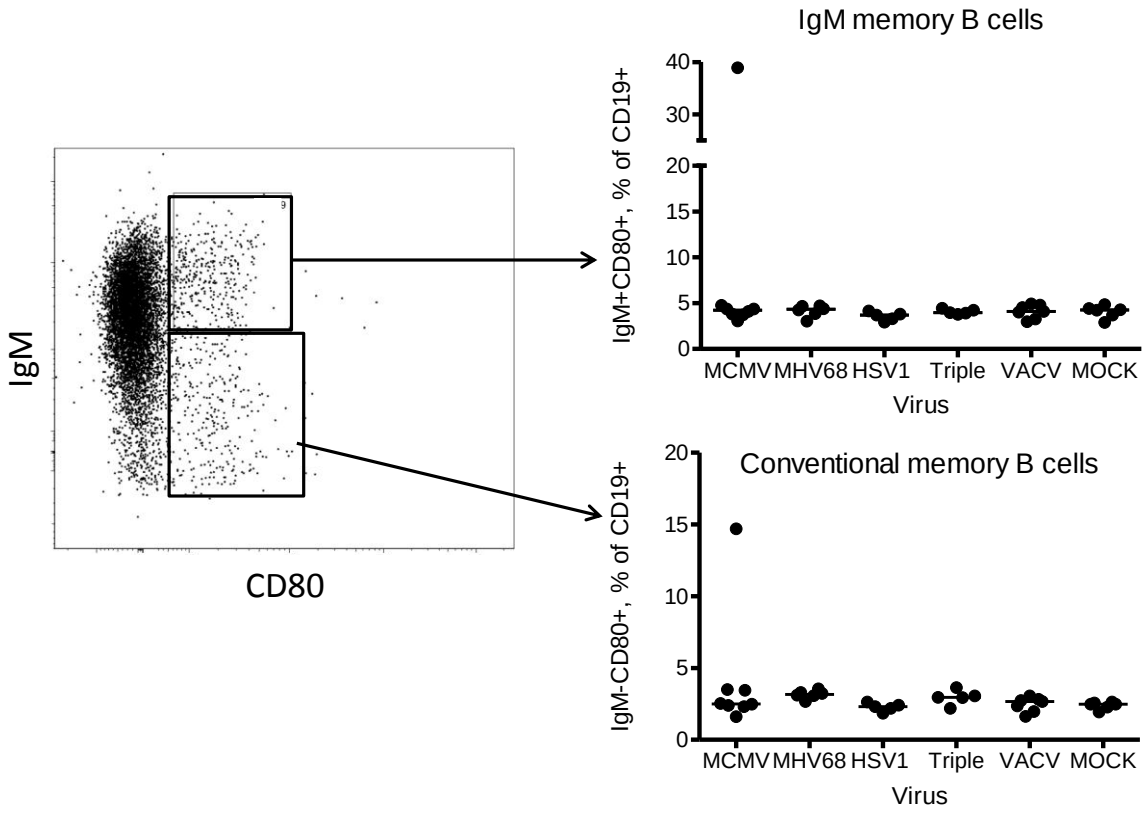

Figure 4.9: Herpesviruses do not affect the frequency of memory B cells. Eight months post priming D2B6 mice were bled and the lymphocytes were stained and analyzed on the expression of CD19+, IgM+/- and CD80+ to define the memory B cell subsets. Each dot represents a mouse and the line is a median of the group. Kruskal-Wallis test followed by Dunns post analysis test was used

4.2 EFFECTS OF HERPESVIRUSES ON THE RESPONSE TO A NEW INFECTION

To determine whether the immune response to new infections is affected in mice carrying herpesviruses, mice were challenged with VSV, a neurotropic virus related to the rabies virus, whose control requires Ig class switch. Weight loss was monitored, and at day 7 and 14 post challenge, the VSV specific T cell response and the VSV

specific immunoglobulin (Ig) titer in herpesviruses infected mice was compared with that from mock-infected mice.

Weight loss and survival was monitored in the group of mice infected with the primary viruses at the age of 6 months and challenged with VSV after 9 months (Figure 4.10A, C) or mice infected at 2 months of age and challenged 15 months later (Figure 4.10B, D). In the first experiment, VSV challenge at nine months after the infection with herpesviruses resulted in no significant difference in weight loss between any herpesvirus-infected group and the mock control (Figure 4.10A). The triple infection group showed more weight loss as compared to the weight loss in the MCMV-infected group which proceeded exactly like in the mock control (Figure 4.10A). On the other hand, MHV-68 infected mice recovered earlier, while the recovery was delayed in mice infected with HSV-1 alone or mice infected with VACV. The mortality showed no significant trends, with one mouse dying on day 2, 5 and 12 in the VACV, HSV-1 and mock group respectively (Figure 4.10C).

In the second experiment, where challenge occurred 15 months post primary infection, the MCMV and the mock had similar kinetic of weight loss, reflecting the previous experiment, but surprisingly the HSV-1 group showed the lowest weight loss (Figure 4.10B). Similarly, in this experiment the triple infected mice showed a more moderate weight-loss than mock-infected controls. MCMV had the lowest survival rate (60%) followed by the MHV-68 group (80%) (Figure 4.10D). The discrepancies between the two experiments might be explained either due to the difference in the age of the mice at primary infection, the length of latent infection, or this might reflect a stochastic

phenomenon that was not associated with the latent viruses the mice were previously infected with.

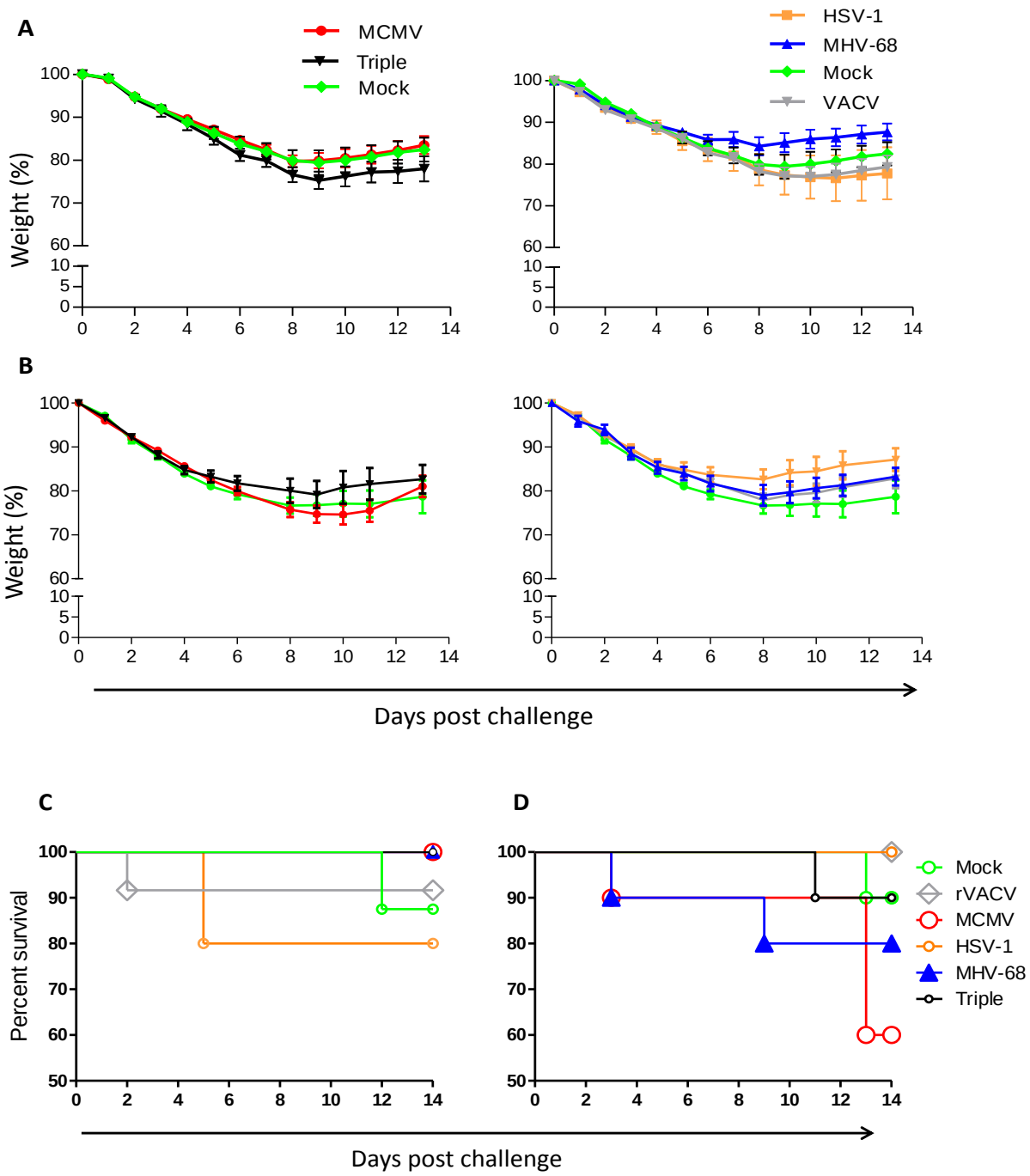

Figure 4.10: Kinetics of weight loss and survival curve of the D2B6 mice. (A, C) Three cohorts of D2B6 mice at the age of 15 months (9 months post primary infection) were all challenged with VSV and then weight loss was monitored until day 13 and survival until day 14. (B, D) Two cohorts of D2B6 mice at the age of 17 months (15 months post primary infection) were all challenged with VSV and then weight loss was monitored until day 13 and survival until day 14

In order to understand the implication of the phenotypic changes observed in this study, mice were sacrificed at day 7 or day 14 after the challenge and compared the adaptive immune response to the VSV from the peripheral blood, spleen or lymph nodes of mice infected with herpesviruses to the control mice infected with non-persistent virus or mock-infected (Figure 4.11).

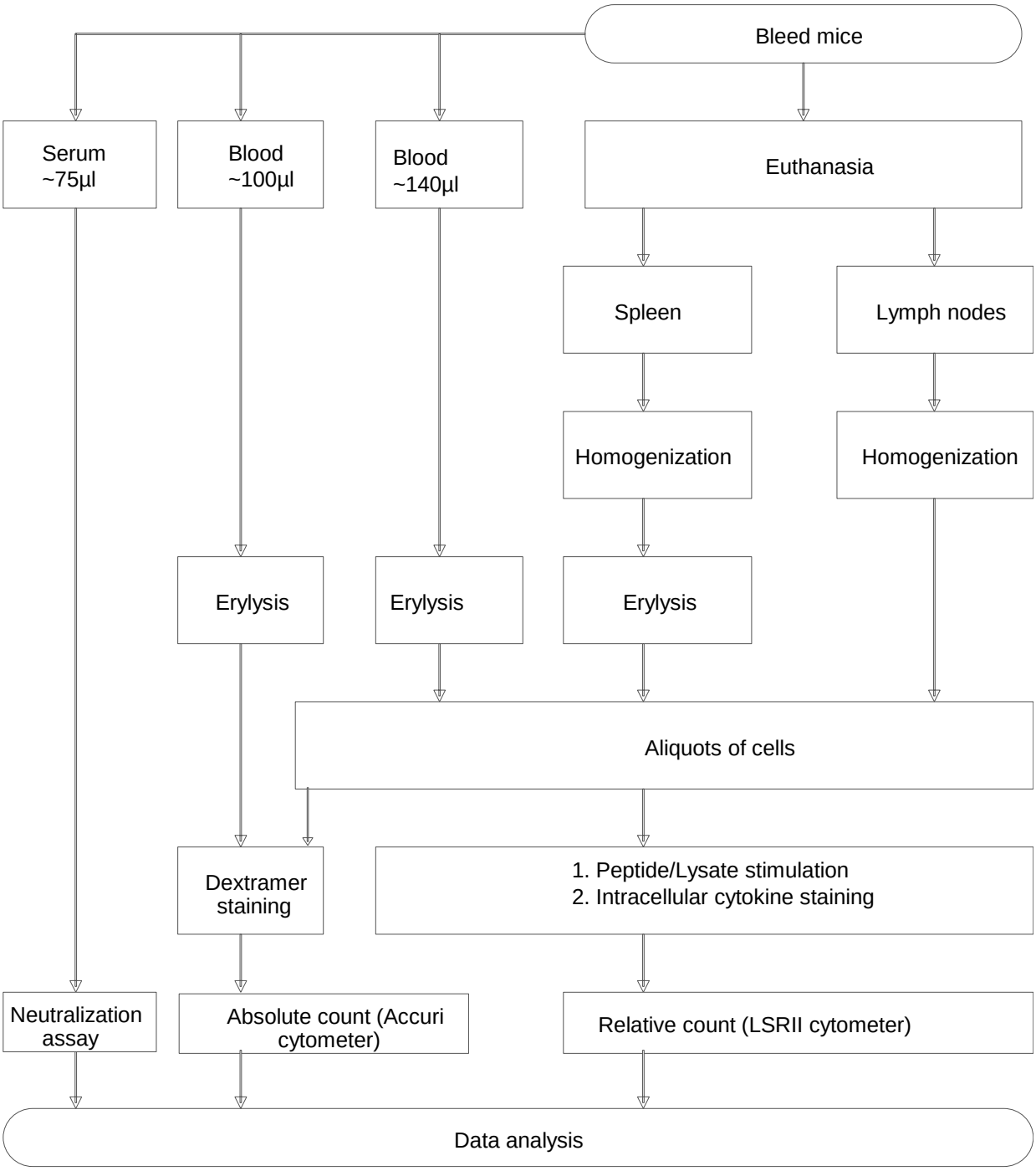

Figure 4.11: A Flow chart diagram to summarize different steps during sample processing

4.2.1 Effects of Herpesvirus infection on CD8 T cell response to VSV

We published recently that the CD8 response to West-Nile virus challenge is diminished in MCMV infected mice, and hence we reasoned that the same may occur in VSV infection. To test this hypothesis, the fraction of CD8 T cells responding by cytokine release to the H-2K^b VSV peptide (RGYVYQGL) was compared after *in vitro* restimulation (Figure 4.12A). Mice were challenged with VSV at the age of 15 months (9 months post primary infection) and assessed the frequency of blood CD8 T cells responding to the VSV at seven days post challenge. Mice carrying latent MCMV or all three herpesviruses showed a significant decrease of CD8 T cells responding to a 6h *in vitro* restimulation with an immunodominant VSV peptide (Figure 4.12B), consistent with our published data (79). Therefore, our data showed that fraction of antigen-responding CD8 T cells in mice latently infected with MCMV, alone or in combination with other viruses (the triple-infected group), are diminished**.** Two weeks post challenge, CD8 T cell responses contracted in the spleen and blood of all the groups, and there were no significant differences between groups. Interestingly, at 14 days post infection, the fraction of VSV-responding cells was significantly higher in the draining lymph node of the triple-infected group, but not in the single-infection with any herpesvirus. Hence these data indicate that contraction of the CD8 T cell responses may be delayed in the draining lymph nodes of mice infected with a combination of the three herpes viruses.

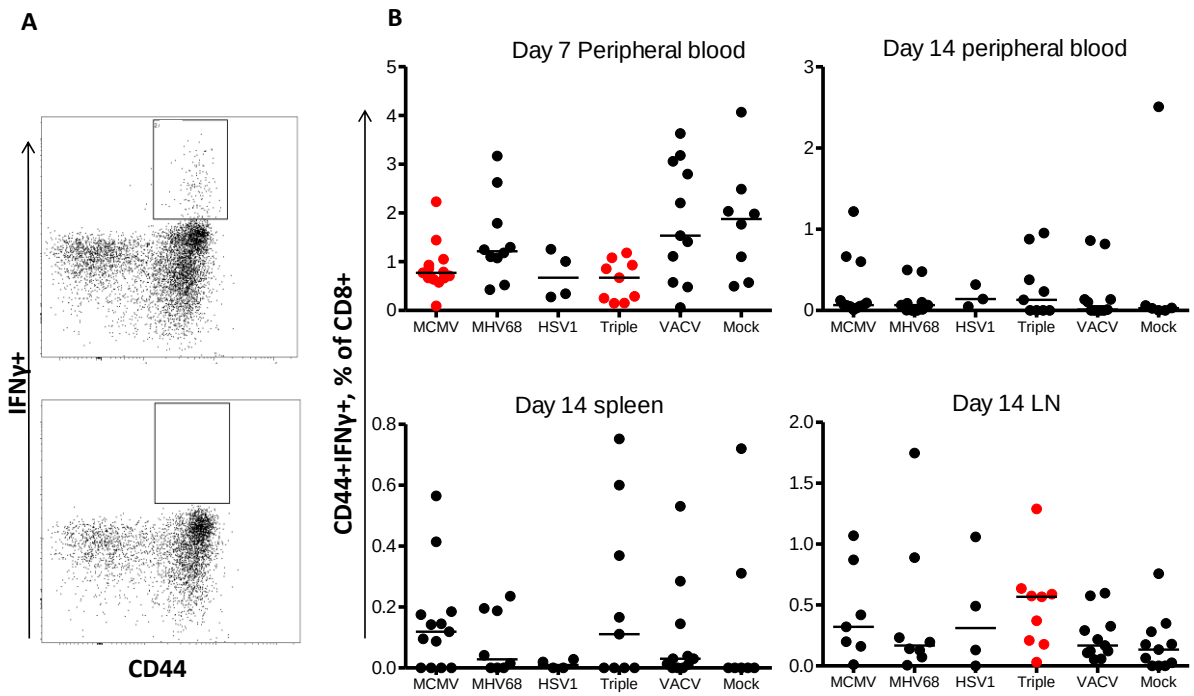

Figure 4.12: Less frequency of responding CD8 T cells to VSV peptide from MCMV infected mice at day seven post challenge. Cells were isolated as shown in figure 4.11 and stimulated with the VSV immonodominant peptide (RGYVYQGL) for 1 hour followed by 5 hours in the presence of Brefeldin A. (A) A representative dot plots showing the percentage of CD8 T cells responding to the peptide stimulation by IFNγ production (upper panel) and negative controls, incubated in the same conditions, but without the peptide (lower panel). (B) CD8 T cell response to VSV from the peripheral blood at day 7 and 14 post challenge was analyzed (upper panel). The lower panels show responses at day 14 from the lymphoid organs (spleen and mediastinal LN) of the same mice. Each dot represent a mouse, the line represent median and the red dots represent groups which had p-value <0.05 when compared to the mock control by using Kruskal-Wallis test followed by Dunns post analysis.

To test if these results may be observed independently of the length of the latent virus infection, analysis of the CD8 response to VSV challenge in D2B6 mice infected at the age of two months, and challenged with VSV after 15 months (at the age of 17 months) was done. Moreover, the analysis was not only in the fraction of VSV-responding CD8 T cells, but also their absolute numbers, as well as the total CD8 count in PBMC

populations and in lymphoid organs. The frequency of VSV specific CD8 T cells in the blood was lower in mice infected with MCMV or all three viruses at 7 and at 14 days post challenge (Figure 4.13 upper panels), although not to a level where the difference would be significant. Interestingly, the absolute number of these cells was essentially identical in all the groups (Figure 4.13 middle panels), despite the differences in the relative counts. The difference between the relative and absolute count of VSV-specific cells could probably be explained by higher total counts of CD8 T cells in herpesvirus-infected mice, which trended up in all groups and was significantly higher in the triple-infected group at day 7 post challenge. (Figure 4.13 lower panel).

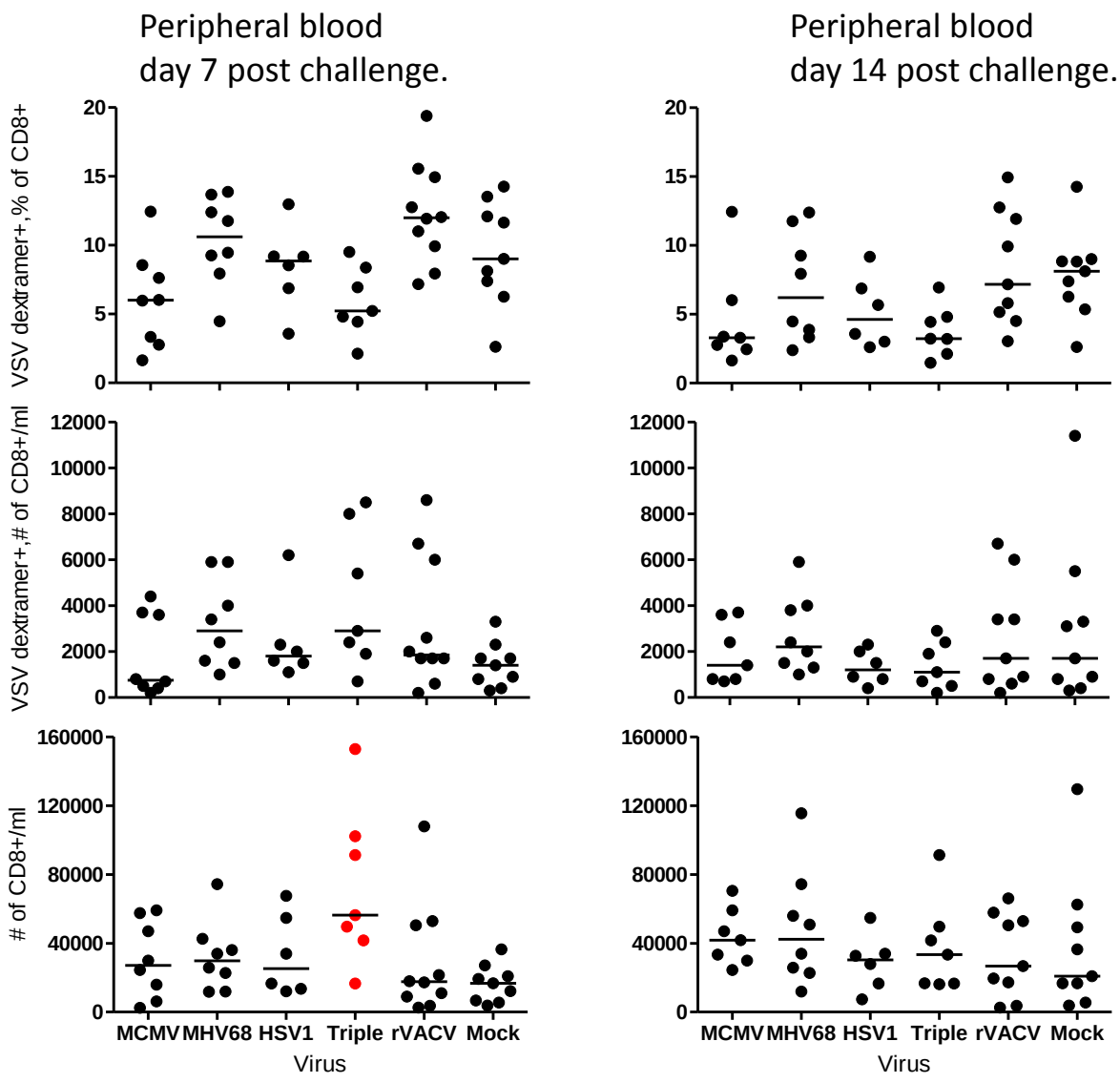

Figure 4.13: Number of VSV dextramer positive and the total peripheral CD8 T cell at day 7 and 14 post challenge. D2B6 mice infected with MCMV, a combination of the three herpesviruses, rVACV or mock-infected at the age of 2 months were challenged with VSV after 15 months. The frequency (upper panel), absolute number (middle panel) of VSV specific CD8 T cells and total CD8 T cells (lower panel) were compared to the mock group. Each dot represent a mouse, the line represents median of the group and the red dots represent groups which showed a statistically significant difference ($p<0.05$, Kruskal-Wallis test followed by Dunns post analysis)

On the other hand, the frequency of VSV specific CD8 T cells and the total CD8 T cells in the draining lymph nodes and spleen at day 14 post challenge was in essence

identical in mice infected with herpesviruses and in the control groups (Figure 4.14 top panels)., while the absolute count of VSV-specific and total CD8 cells was slightly elevated (Figure 4.14 middle and bottom panels).

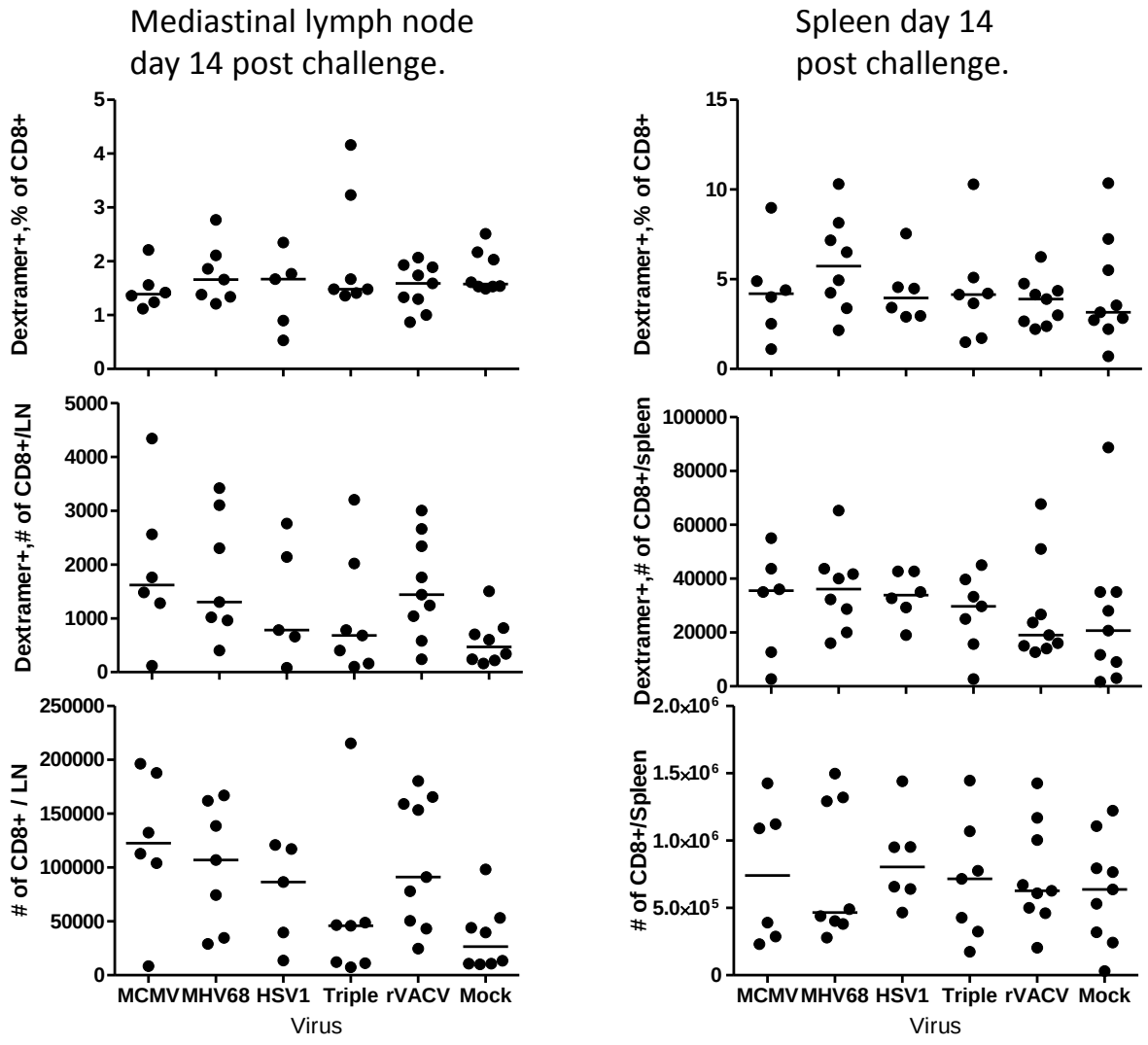

Figure 4.14: Numbers of VSV dextramer positive and the total CD8 T cell at day 14 post challenge from the lymphoid organs. D2B6 mice infected with MCMV, a combination of the three herpesviruses, rVACV or mock-infected at the age of 2 months were challenged with VSV after 15 months. The frequency (upper panel), absolute number (middle panel) of VSV specific CD8 T cells and total CD8 T cells (lower panel) were compared to the mock group. Each dot represent a mouse, the line represents median of the group (Kruskal-Wallis test followed by Dunns post analysis)

Therefore, in mice infected for a longer period of time there was similar reductions in the fractions of responder cells, but the extension of our analysis to include absolute counts of responding cells revealed, unexpectedly, that the responses were not decreased in absolute terms in the peripheral blood or in other organs.

4.2.2 The time from primary infection with herpesviruses to the challenge is not crucial for the homeostatic or functional changes occuring neither in the T nor the B cell compartment

To validate if the results obtained with the D2B6 (F1) mice were generally applicable to other mouse strains or specific for this mouse strain, similar experiments in 129S2/SvPasCrl x BALB/cAnNCrl F1 mice (129B) were performed. Moreover, to test if the length of time from herpesvirus infection till the encounter with a new unrelated infection affects the strength of the immune response, mice from the same litter were primed with herpesviruses early or later in their life, and challenged with VSV at the age of 18 months (Figure 4.15)**.**

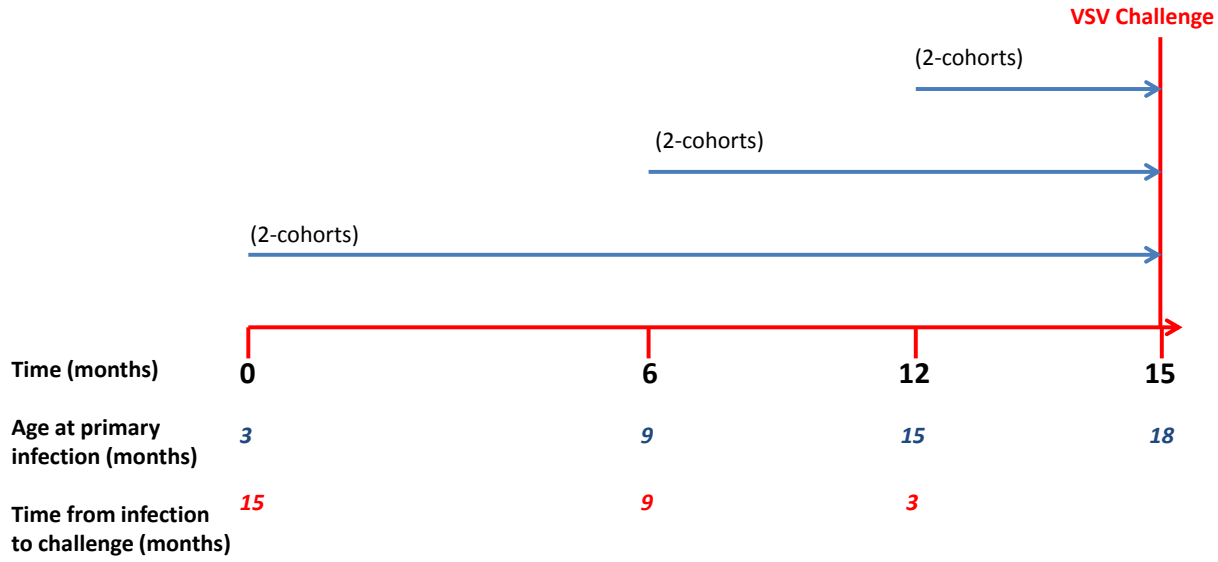

Figure 4.15: *In vivo* experimental plan. 129B mice were infected intraperitoneally at 3, 9, 15 or 18 months of age with individual herpesviruses (i.e. MCMV and MHV-68), or their combination. Controls were infected with rVACV, influenza (flu) virus, or mock infected. Each experimental group consisted of at least five mice and all experiments were independently repeated to confirm results. At the age of 18 months, all the mice were intranasally challenged with VSV and 7 days post challenge blood and lymph nodes were analyzed for immune response to VSV.

The blood fraction of VSV specific CD8 T cells was compared in mice challenged with VSV at 7 days, 3 months or 9 months post infection with herpesviruses. To evaluate the fraction of antigen-specific cells in total and the fraction that was functionally active, half of the blood samples were stained with peptide-MHC multimers, whereas the other half was subject to a functional assay by peptide in vitro restimulation and intracellular IFNγ staining, using the same peptide as the one used in the multimer. Consistent with our previous results, the mice infected with MCMV showed significantly lower frequencies of antigen specific cells (Figure 4.16 upper panels), and this could be observed at all times tested. Similarly, mice infected with MHV-68 and MCMV in combination showed lower fractions of dextramer positive cells at three or nine months post primary infection (this

condition was not tested in mice infected for one week). Functional assays showed similar trends: The CD8 T cell response to stimulation with the VSV peptide was significantly lower in VSV challenged mice at one week post MCMV infection, while the median responses were lower, but not significantly, in the cohorts of mice challenged at three or nine months post MCMV infection (Figure 4.16 middle panels). On the other hand, the fraction of CD8 T cells with the EM phenotype was significantly higher in the MCMV and in the double-infected group, and this was significant in mice challenged at 7 days or three months post primary infection, while a similar, but non-significant trend was observed at nine months post infection, Interestingly, rVACV infection resulted in a significantly higher fraction of the EM CD8 T cells compared to the mock group at 7 days post infection, although this was not the case in mice infected with influenza (Figure 4.16 lower panels).

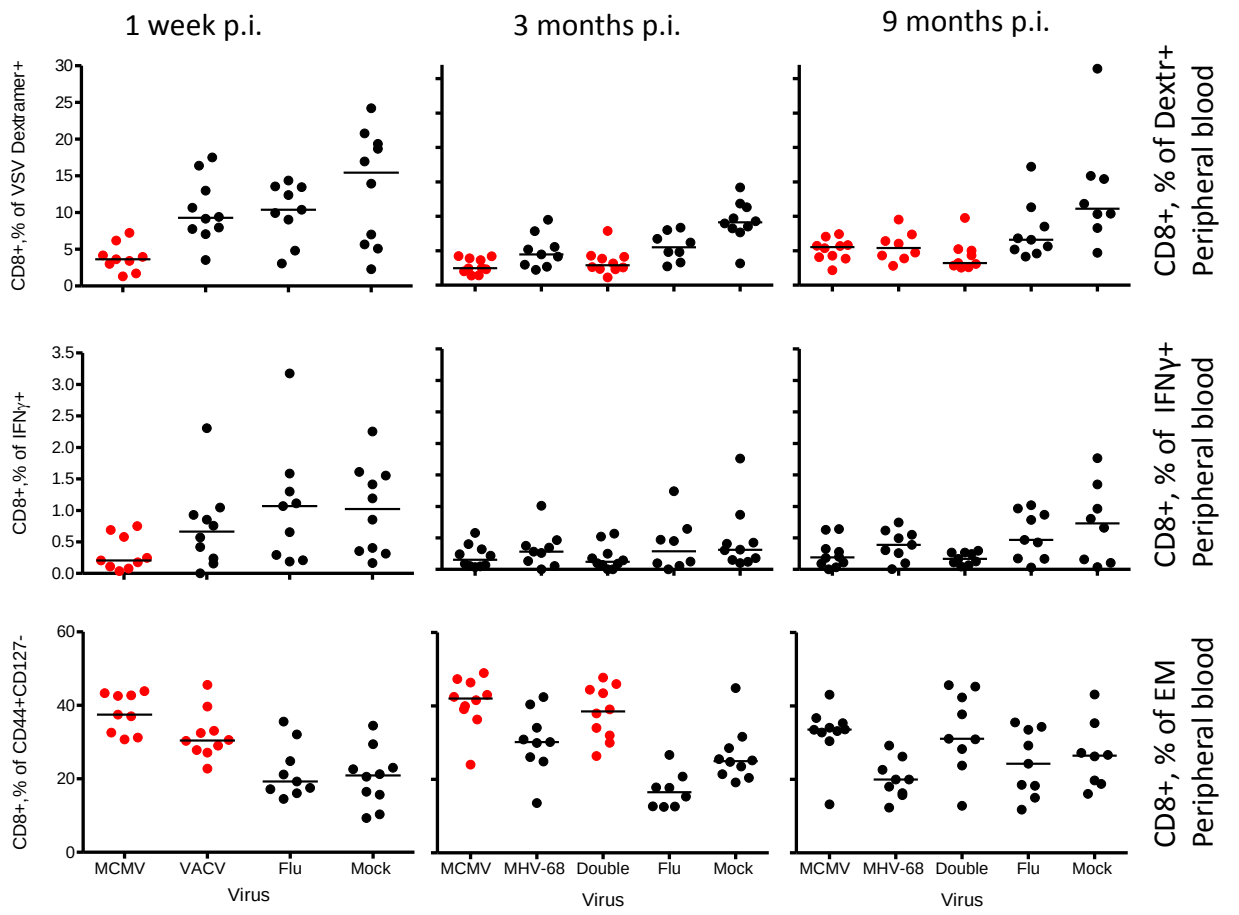

Figure 4.16: CMV infection results in low frequency of CD8 T cells responding to VSV, but elevated frequency of CD8 EM, regardless of the time of challenge. 129B Mice infected with MCMV, MHV-68, combination of MCMV and MHV-68 (Double), rVACV, Flu or mock infected at the age of 9, 15 and 18 months were all challenged with VSV at the age of 18 months. Seven days post challenge; the fraction of CD8 T cells specific for the VSV dextramer (upper panels), CD8 T cells responding to the VSV peptide (middle panels) and the fraction of the CD8 EM (bottom panels) were compared to the mock group. Each dot represents a mouse and the red dots represent groups which showed a statistically significant difference ($p<0.05$, Kruskal-Wallis test followed by Dunns post analysis)

4.2.3 Herpesvirus infection does not affect the absolute number of CD8 T cells responding to VSV challenge

While the percentage of VSV responding cells was consistently lower in mice infected with MCMV, it was not clear if this would be matched by a similar decrease of the response in absolute terms. Therefore, the absolute responses to VSV peptides were evaluated in the same cohorts shown in the previous figure. Interestingly, the absolute number of VSV specific CD8 T cells measured by dextramer staining or *in vitro* VSV peptide stimulation from mice infected with the herpesviruses was comparable to the mock group regardless of the time at which the primary infection was done (Figure 4.17 upper and middle panels). The total number of blood CD8 T cells in the same mice was significantly higher in the groups of mice infected with MCMV compared to the mock group, while the MHV-68 group and the double infection group had significantly higher numbers in the cohort of mice challenged three months post infection (Figure 4.17 lower panels). Therefore, the decrease in the percentage of CD8 cells that are specific for the VSV antigen (Figure 4.16) was not caused by the decrease of their count, but rather by the increase of the size of the CD8 pool (Figure 4.17), and mainly by the huge increase of the EM subset of CD8 cells (Figure 4.16, lower panels).

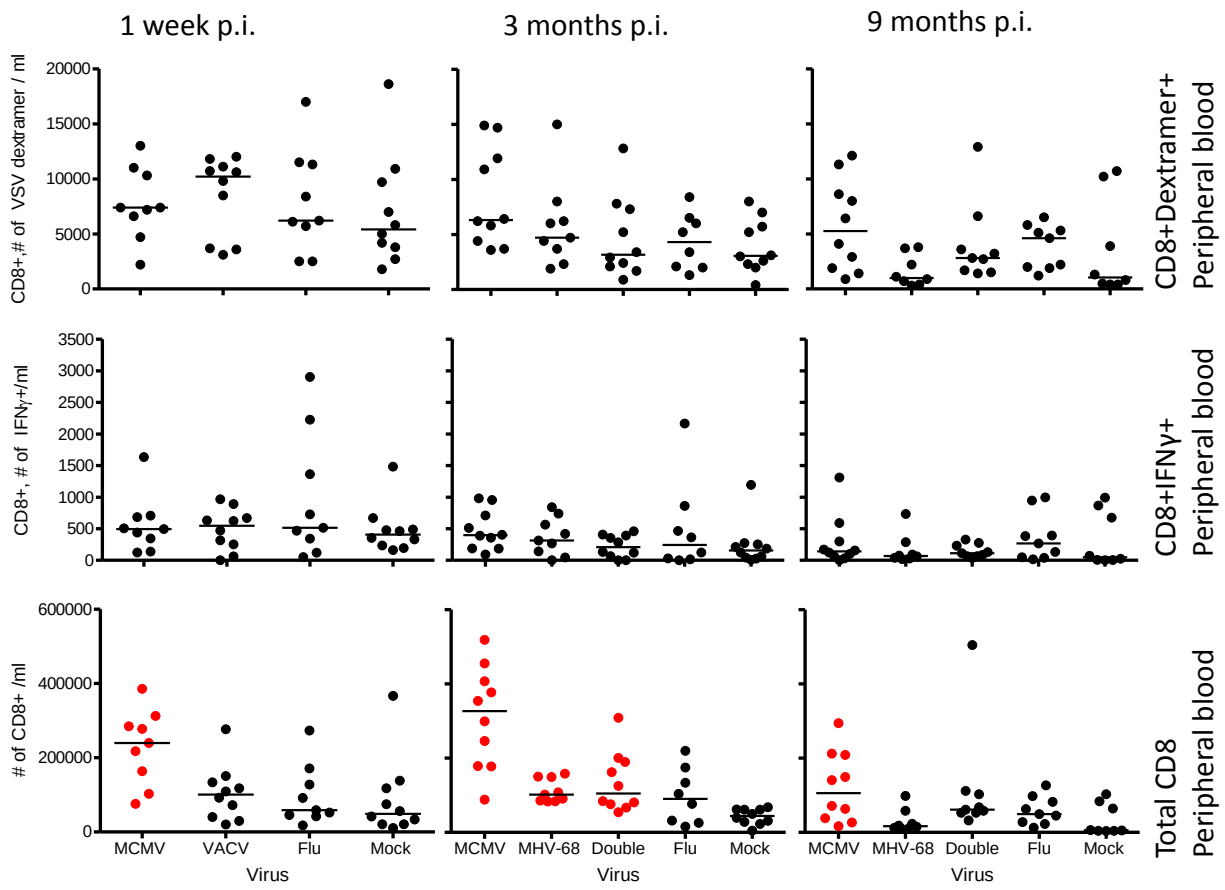

Figure 4.17: Latent infection with herpesviruses does not affect the absolute count of the VSV specific CD8 T cells. 129B Mice infected with MCMV, MHV-68, combination of MCMV and MHV-8 (double), rVACV, Flu or mock infected at the age of 9, 15 and 18 months were all challenged with VSV at the age of 18 months. Seven days post challenge; the absolute number of VSV dextramer positive CD8 T cells (upper panel), CD8 T cells responding to the VSV peptide (middle panel) and the total CD8 T cells (lower panels) were compared to the mock group. Each dot represents a mouse and the red dots represent groups which showed a statistical significant difference ($p<0.05$, Kruskal-Wallis test followed by Dunns post analysis)

4.2.4 Herpesvirus infection does not impact the frequency of functionally active CD8 T cells within the pool of CD8 T cells that are specific for the VSV antigen

Since the fractions of dextramer specific CD8 T cells were significantly smaller, but a less pronounced decrease of the cell populations that responded to the antigen by IFNγ, raised a possibility that herpesviruses might affect the fraction of CD8 T cells responding to the VSV peptide within the antigen-specific population. Therefore, the fraction of CD8 T cells which were functionally active (producing IFNγ after in vitro peptide stimulation) was analyzed within the pool of dextramer positive cells, since the same peptide used for the stimulation was a constituent of the MHC-peptide complexes on the dextramer molecule. The fraction of functional cells was similar in all experimental groups and controls (Figure 4.18). While in this study analysis of other properties of these cells (e.g. degranulation activity, cytotoxicity, or proliferative capacity) were not done, these data suggested that herpesviruses do not affect the functional cytokine responses to antigen stimulation of CD8 T cells generated against a new viral infection.

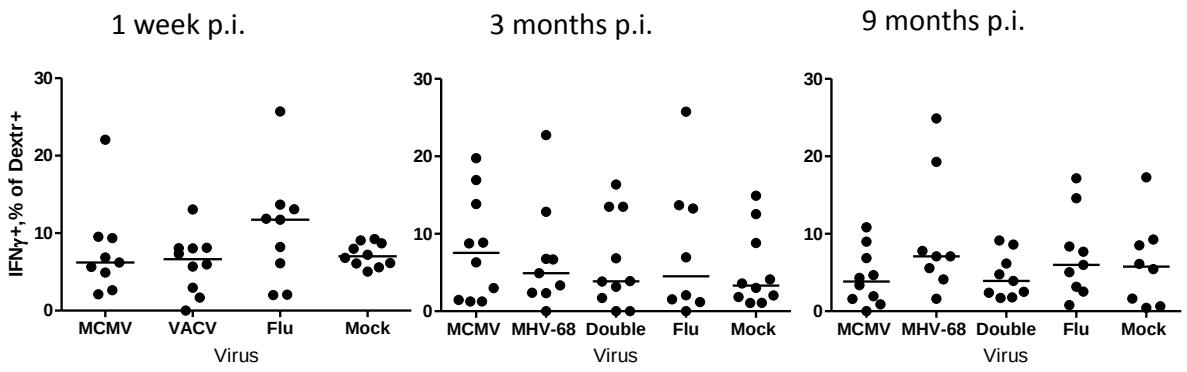

Figure 4.18: Latent herpesvirus infection does not affect the frequency of CD8 T cells responding to the *in vitro* peptide stimulation within the VSV specific cells. 129B Mice infected with MCMV, MHV-68, combination of MCMV and MHV-68 (Double), rVACV, Flu or mock infected at the age of 9, 15 and 18 months were all challenged with VSV at the age of 18 months. Seven days post challenge; the fractions of CD8 T cells responding to the VSV peptide within the dextramer positive population were analyzed (Kruskal-Wallis test followed by Dunns post analysis). Each dot represents a mouse

4.2.5 The increase in the absolute number of blood CD8 T cells in MCMV infected mice is a result of the increase in the number of EM cells in the peripheral blood but not in lymph nodes.

The enlargement of the blood CD8 T cell pool in MCMV infected mice raised the question about the provenance of these cells. While it was likely that the relative increase of the EM subset in the peripheral blood (see Figure 4.16 lower panels) may have affected the size of the total CD8 pool, it remained unclear if this would be restricted to the blood pool, or a result of an increase of the EM subset in the sites where the adaptive immune response is initiated, namely in the draining lymph nodes. The absolute number of CD8 T cells with EM phenotypes (defined as CD127-CD44+) in the peripheral blood was significantly higher in the group of mice which were previously infected with MCMV and challenged with VSV either one week, three or nine months,

and similar results were observed in the double infected groups (Figure 4.19 upper panel). The absolute number of EM CD8 T cells in the mediastinal (draining) lymph node was essentially identical in all the tested conditions (Figure 4.19 middle panels). Likewise, the size of the EM pool was not changed in inguinal (non-draining) lymph nodes of any mouse group (Figure 4.19 lower panels). On the other hand, the EM pool was larger in draining than in non-draining lymph nodes (Figure 4.19 middle and lower panels), strongly arguing that VSV infection activated the CD8 cells specifically in the draining lymph node. Therefore, our results argue that the increases in the EM CD8 T cells in peripheral blood were unlikely a result of cells responding to the VSV challenge, but rather were a reflection of the CMV specific induction of EM cells (please see also Figure 4.2).

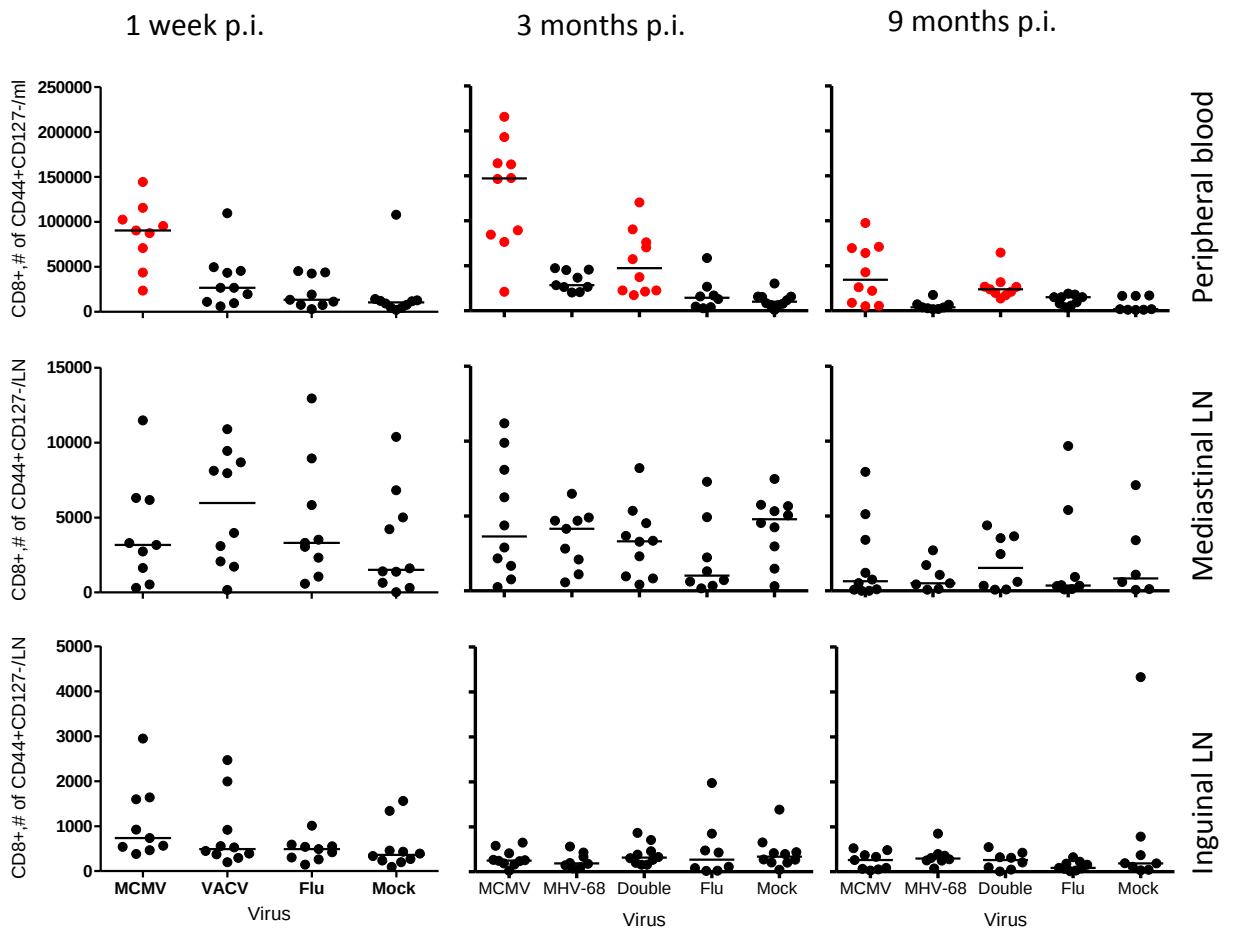

Figure 4.19: The increase in the absolute count of total peripheral CD8 T cells is a result of an increase in the absolute number of the EM subsets. 129B Mice infected with MCMV, MHV-68, a combination of both MCMV and MHV-8 (double), rVACV, Flu or mock infected at the age of 9, 15 and 18 months were all challenged with VSV at the age of 18 months. Seven days post challenge; the absolute number of CD8 T cells from the peripheral blood and lymph nodes with EM phenotype were compared to the mock group. Each dot represent a mouse and the red dots represent groups which showed a statistical significant difference ($p<0.05$, Kruskal-Wallis test followed by Dunns post analysis)

While the EM pool was elevated in the blood of MCMV-infected mice, this did not exclude the possibility that other CD8 subsets were also elevated. Three or nine months post infection, the number of CD8 T cells with CM phenotype (CD127+CD44+) in the

peripheral blood of MCMV or double-infected mice was significantly higher than in mock-infection controls (Figure 4.20 upper panels). Similar results were obtained in the group that was primed with MHV-68 and challenged with VSV three months later. In the MCMV group challenged with VSV a week post primary infection there was a similar trend, but it did not reach significance. On the other hand, there were no significant differences in the size of the CM pool in lymph nodes. (Figure 4.20 middle and lower panels). Finally, the absolute number of naïve (CD44-CD127+) CD8 T cells in the peripheral blood and lymph nodes of the same mice were assessed.

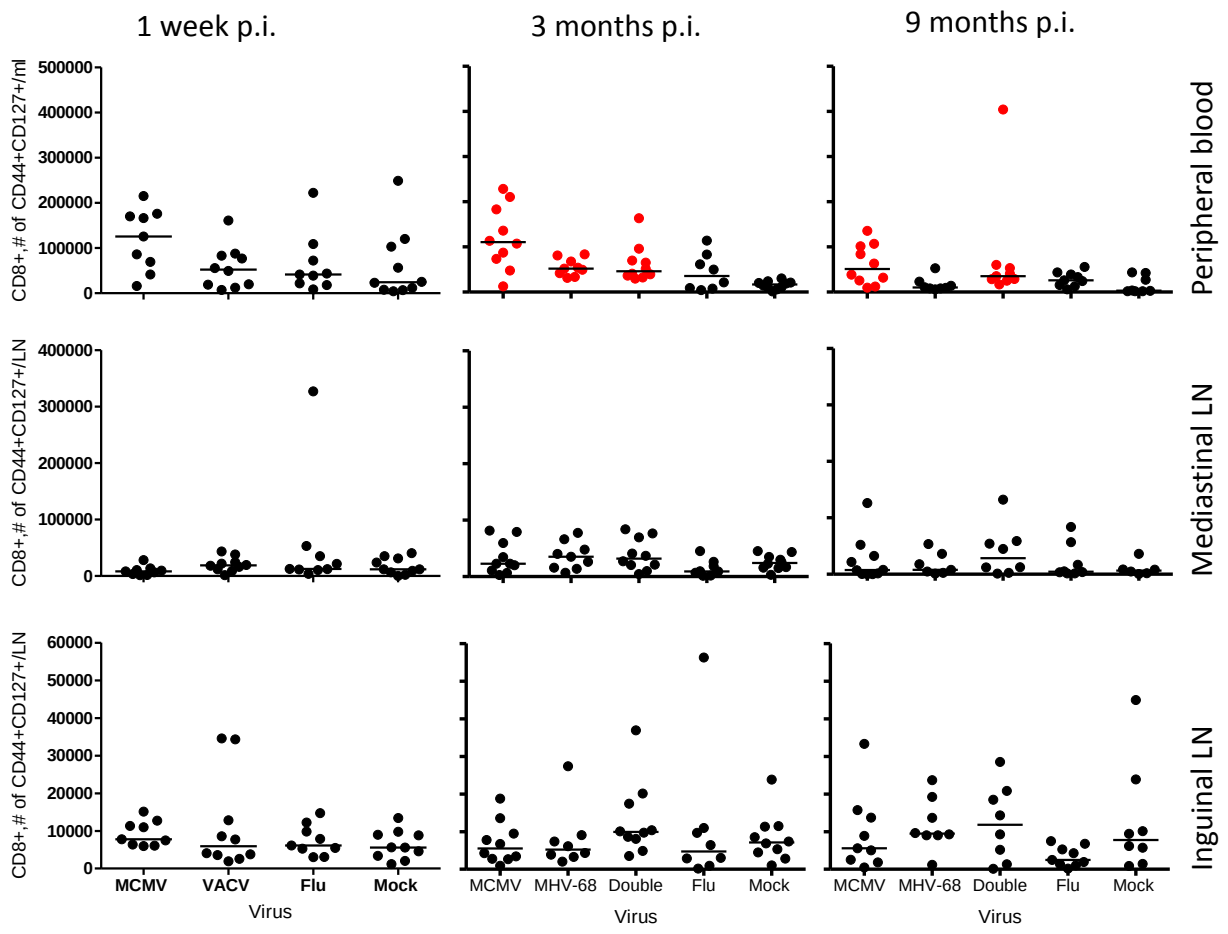

Figure 4.20: The increase in the absolute count of total peripheral CD8 T cells is as a result of an increase in the absolute number of the CM subsets. 129B Mice infected with MCMV, MHV-68, a combination of both MCMV and MHV-8 (double), rVACV, Flu or mock infected at the age of 9, 15 and 18 months were all challenged with VSV at the age of 18 months. Seven days post challenge; the absolute number of CD8 T cells from the peripheral blood and lymph nodes with CM phenotype were compared to the mock group. Each dot represent a mouse and the red dots represent groups which showed a statistical significant difference ($p<0.05$, Kruskal-Wallis test followed by Dunns post analysis)

Interestingly, the numbers of naïve blood CD8 cells were higher in mice infected with MCMV, and this was significant in mice infected for 7 days or three months prior to challenge, (Figure 4.21 upper panels). There was no significant difference in the

absolute number of the naïve CD8 T cells in the draining or non-draining lymph nodes in any experimental group (Figure 4.21 middle and lower panels).

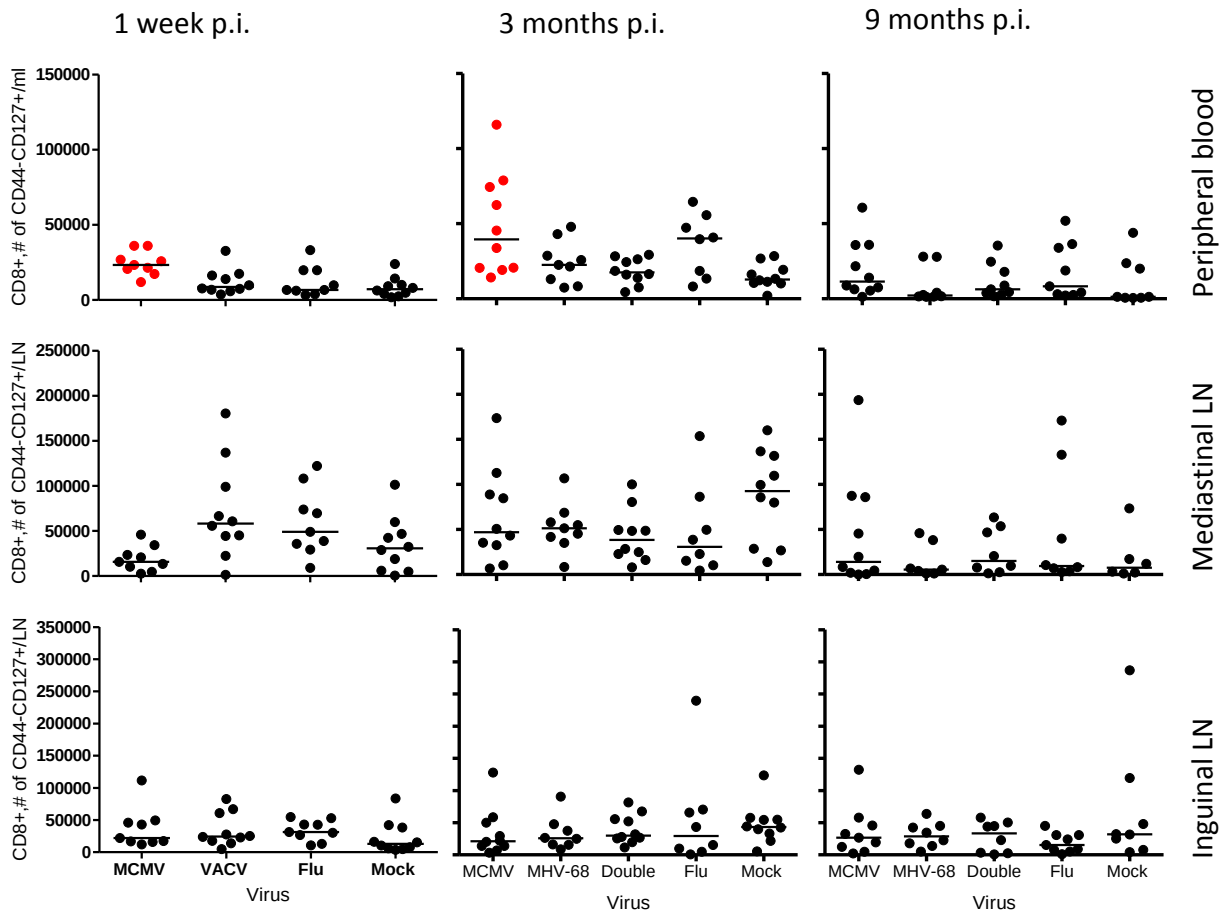

Figure 4.21: The absolute number of peripheral naïve CD8 T cells is increased in MCMV infected mice. 129B Mice infected with MCMV, MHV-68, a combination of both MCMV and MHV-8 (double), rVACV, Flu or mock infected at the age of 9, 15 and 18 months were all challenged with VSV at the age of 18 months. Seven days post challenge; the absolute number of CD8 T cells from the peripheral blood and lymph nodes with a naïve phenotype were compared to the mock group. Each dot represent a mouse and the red dots represent groups which showed a statistical significant difference ($p<0.05$, Kruskal-Wallis test followed by Dunns post analysis)

4.2.6 Persistent herpesvirus infection does not affect the total number of CD8 T cells and absolute number of VSV specific CD8 T cells in the draining and non-draining lymph nodes

Our results argued that MCMV infection does not alter the number of VSV specific cells in the peripheral blood, but that it masquerades as a decrease of the percentage of VSV-responding cells in the CD8 pool, because it increases the total number of CD8 cells. It remained unclear if herpesvirus infections altered the relative or absolute response to VSV in other lymphatic organs. Therefore, the effects of herpesvirus infection on the number of total CD8 T cells and VSV specific cells in draining lymph nodes were tested. No infection condition altered the size of the CD8 pool or of the VSV-dextramer positive subset in the lymph nodes of mice challenged with VSV at nine months post primary infection (Figure 4.22).

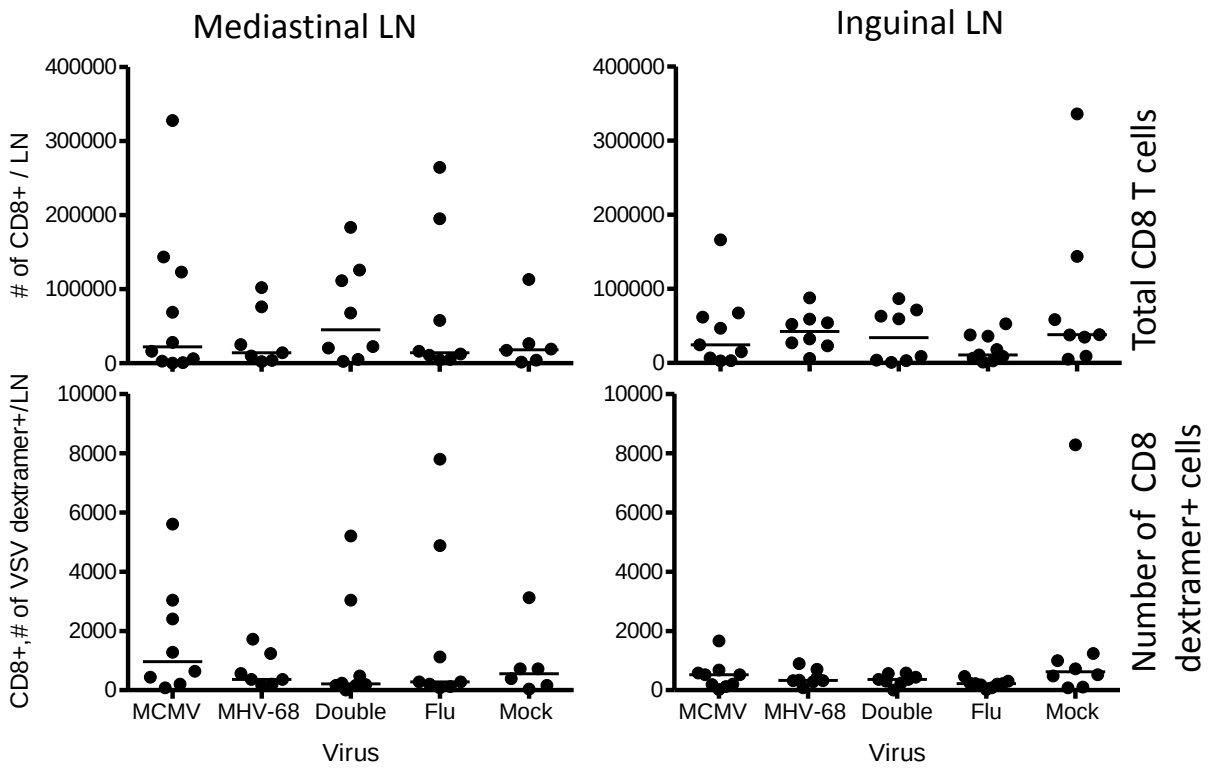

Figure 4.22: The absolute count of total CD8 and VSV-specific CD8 T cells in the draining and non-draining lymph nodes after VSV challenge is not affected by latent herpesvirus infection. Nine months post herpesvirus (or control) infection, mice were challenged with VSV and the absolute number of total CD8 T cells and VSV-specific CD8 T cells was defined by flow cytometric analysis in an Accuri cytometer. Each dot represents a mouse, horizontal lines are medians. Groups were tested by Kruskal-Wallis test followed by Dunns post analysis.

4.2.7 Herpesvirus infection does not affect the CD4 T cell response to VSV

To find out if the CD4 T cell compartment may be affected with any herpesvirus infection, this study compared the fraction of CD4 T cell responding to the VSV cell lysate by secreting cytokines in mice latently infected with herpesviruses or with control non-persisting viruses to the mock-infected control mice. Mock infected lysate was used

as the negative control for the stimulation, to assess the amount of TNFα secreting cells in absence of antigen-specific responses (Figure 4.23A lower dot plot) and their percentage was subtracted from the fraction of CD4 T cells responding to the VSV lysate (Figure 4.23A upper dot plot). The following cytokines were tested: interferon gamma (IFNγ), tumor necrosis alpha (TNFα), Interleukin-2, Interleukin 4 and interleukin 17 responses to lysate stimulation, and only TNFα offered responses that could be readily interpreted, whereas all other cytokine responses could not be efficiently separated from the background noise (data not shown). Therefore the focus was on TNFα responses and CD4 T cell responses were analyzed in both D2B6 and 129B strains of mice. There was no significant difference in the fraction of VSV-specific cells neither at day 7 in the peripheral blood nor at day 14 post challenge in the draining lymph nodes and spleens of D2B6 mice latently infected with any herpesvirus as compared to the control groups (Figure 4.23B, upper panels).

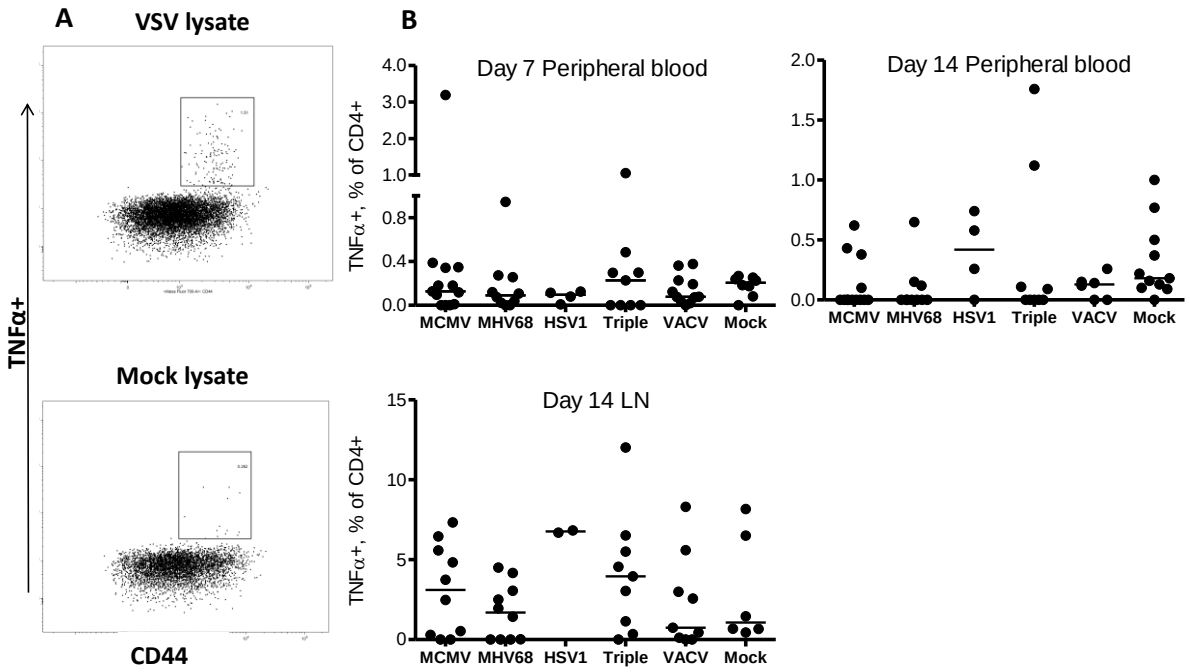

Figure 4.23: Herpesviruses do not affect CD4 T cell response to VSV in the D2B6 mice. (A) Seven and 14 days after the VSV challenge CD4 T cells from the peripheral blood and the draining lymph nodes from the D2B6 mice were stimulated with either VSV lysate or mock lysate (upper and lower panel respectively) for one hour followed by six hours stimulation in the presence of BFA. (B) The fractions of VSV specific CD4 T cells from mice previously infected with the herpesviruses (infected for 9 months) were compared to the same cells from the peripheral blood of mock infected mice (Kruskal-Wallis test followed by Dunns post analysis)

Similar results were observed in cells from the peripheral blood and lymph nodes of 129B mice (Figure 4.24). However, in the blood of 129B mice there was a non-significant trend towards higher fraction of peripheral VSV specific CD4 T cells from mice primed with MCMV, both herpesviruses or flu for 9 months (Figure 4.24 upper panels), whereas in D2B6 mice this could be observed in the draining lymph node (LN), but not in the blood (Figure 4.23B). The analysis of absolute response revealed a similar, but even more pronounced trend in the blood of the 129B mice but again the

difference was not statistically significant (Figure 4.25). Absolute counts of CD4 responses in D2B6 mice were not assessed due to historical reasons.

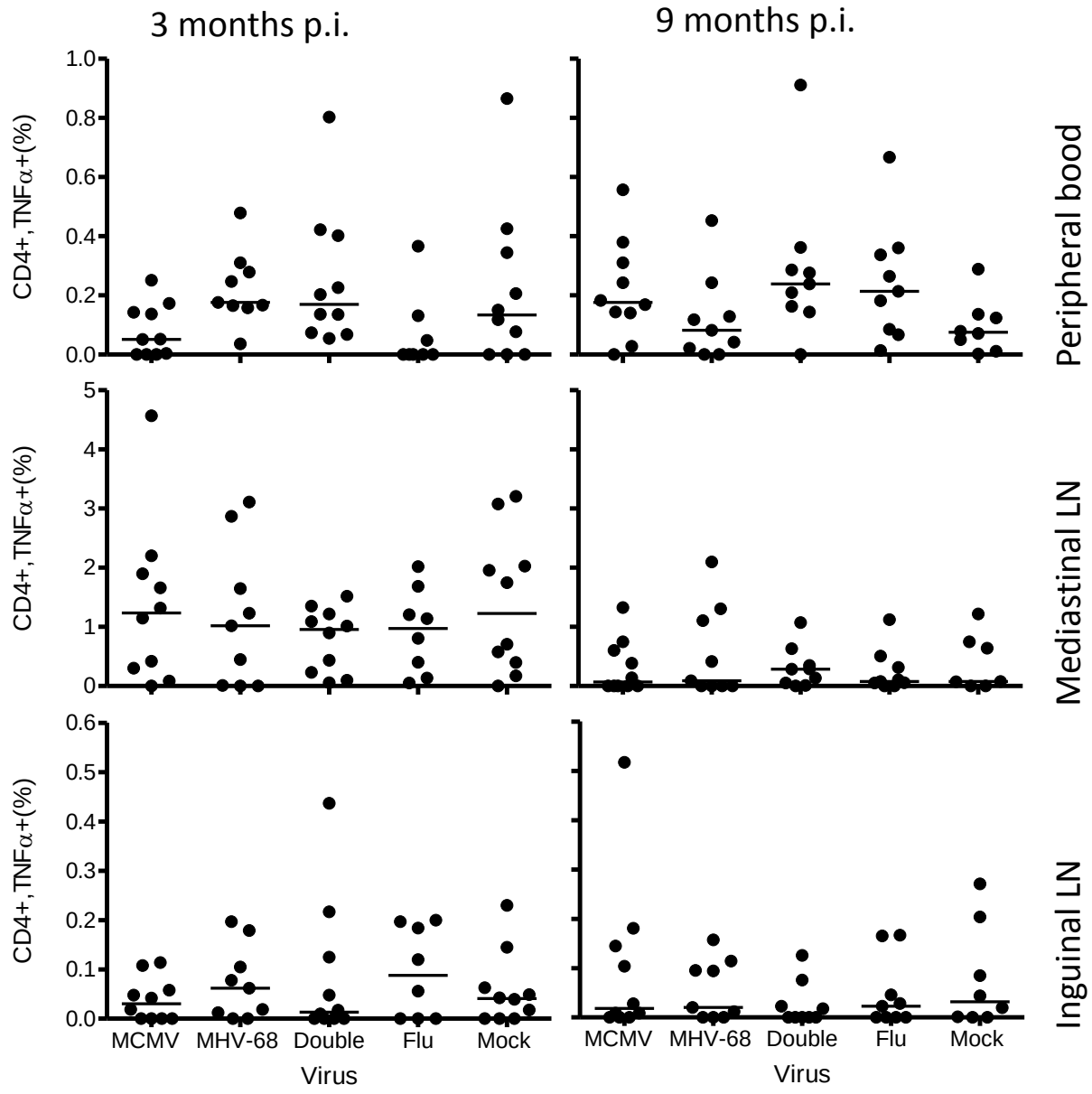

Figure 4.24: Herpesviruses do not affect CD4 T cell response to VSV in the 129B mice. Seven days after the VSV challenge fractions of VSV specific CD4 T cells from 129B mice previously infected with herpesviruses were compared to the same cells from the peripheral blood and lymph nodes of mock infected mice. Each dot represents a mouse (Kruskal-Wallis test followed by Dunns post analysis)

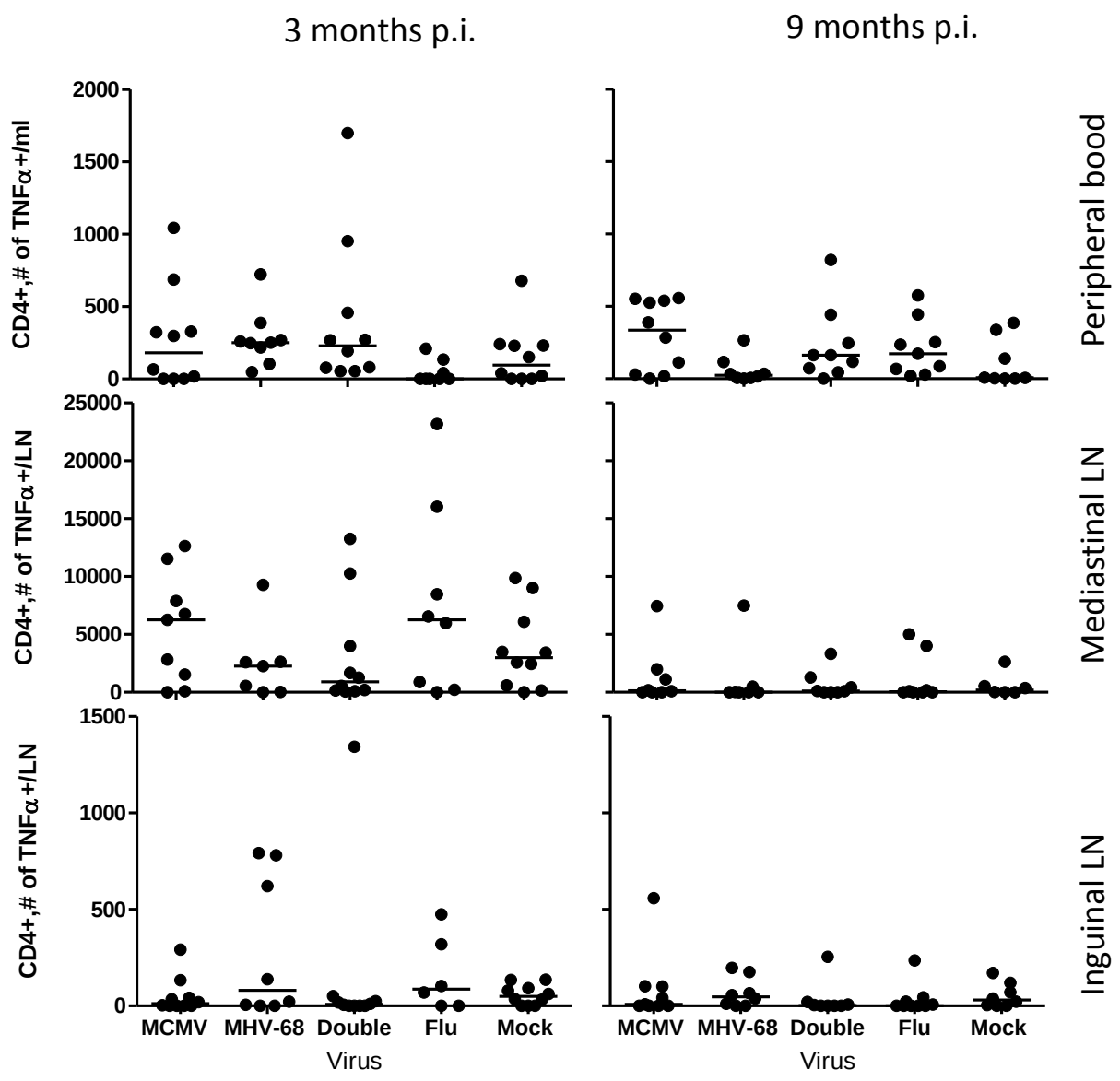

Figure 4.25: Herpesviruses do not affect the absolute number of VSV specific CD4 T cell in the 129B mice. Seven days after the VSV challenge the absolute number of VSV specific CD4 T cells from 129B mice previously infected with herpesviruses were compared to the same cells from the peripheral blood and lymph nodes of mock infected mice. Each dot represents a mouse (Kruskal-Wallis test followed by Dunns post analysis)

4.2.8 Effects of Herpesvirus infection on the VSV specific neutralizing immunoglobulin titer

VSV can be controlled by neutralizing antibodies produced by plasma cells (138); however, in order to mount a protective humoral response, the IgG isotype class switch must take place. Therefore, this study also wanted to ascertain the class switch of VSV-specific antibodies in mice that are latently infected with herpesviruses, and evaluate their functional capacity. To this end, sera collected at day seven or fourteen post-VSV challenge were used in an *in vitro* neutralization assay to determine the neutralization capacity of VSV specific antibodies. In D2B6 mice there was no significant difference in the neutralizing titer of the total Ig (IgG+IgM) serum fraction (Figure 4.26A). The virus-neutralization capacity of β-mercaptoethanol treated sera was tested. Since β-mercaptoethanol disrupts the IgM antibodies, these sera contained only the antibodies that switched their class. Interestingly, the MCMV-infected group showed a significantly lower neutralizing titer in the IgG fraction, as compared to the mock group at day seven post challenge. Since this difference could not be observed at day fourteen-post challenge (Figure 4.26B), this result suggested a delayed immunoglobulin class switch in mice infected with MCMV.

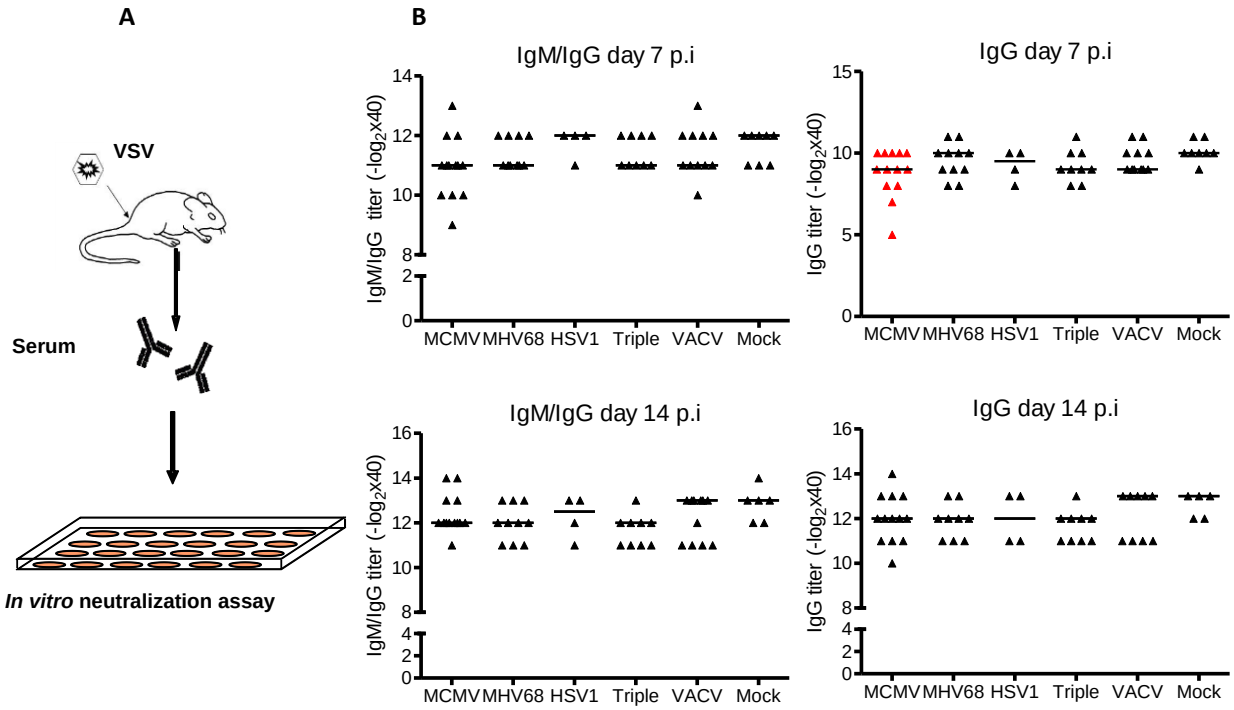

Figure 4.26: MCMV infection results in a delayed VSV specific IgG class switch in D2B6 mice. (A) Neutralization assay was done using the sera from the D2B6 mice challenged with VSV for 7 or 14 days. Serum was pre diluted 40 fold followed by 1:2 serial dilution steps. (B) The VSV specific immunoglobulin titer was represented as the serum dilution at which the number of VSV plaques was reduced by 50%. Each triangle represents a mouse and the group with red symbols had a $p < 0.05$ (Kruskal-Wallis test followed by Dunns post analysis).

The same assay was repeated in the 129B strain of mice infected with the primary viruses for two weeks, three months or fifteen months prior to the VSV challenge. In contrast to the previous experiment, here blood was collected from the mice at days four and seven after the VSV challenge, in order to analyze very early responses. In line with results from D2B6 mice, the total Ig titer from mice infected with herpesviruses showed no difference in neutralizing capacity to the sera from mock-infected mice and

this was the case at either time point (Figure 4.27, 4.28, top panels). Moreover, the IgG fraction showed absolutely no VSV neutralization at day four post infection (Figure 4.27, lower panels), consistent with the idea that neutralization by class-switched antibodies requires more time, but also indicating that VSV-neutralization by β-mercaptoethanol treated sera from day 7 and 14 post infection (see Figure 4.26B, right panels) reflected the increase in the Ag-specific antibody IgG titer. At day seven post challenge the total immunoglobulin in the sera from mice previously infected with viruses was comparable to the mock control group, but again there was a trend towards lower IgG responses in herpesvirus infected mice. Namely, the VSV specific IgG titer was significantly lower in the sera from mice latently infected with the combination of MHV-68 and MCMV (Figure 4.28) and similar trends, although not significant ones, were observed in other groups infected with MCMV alone.

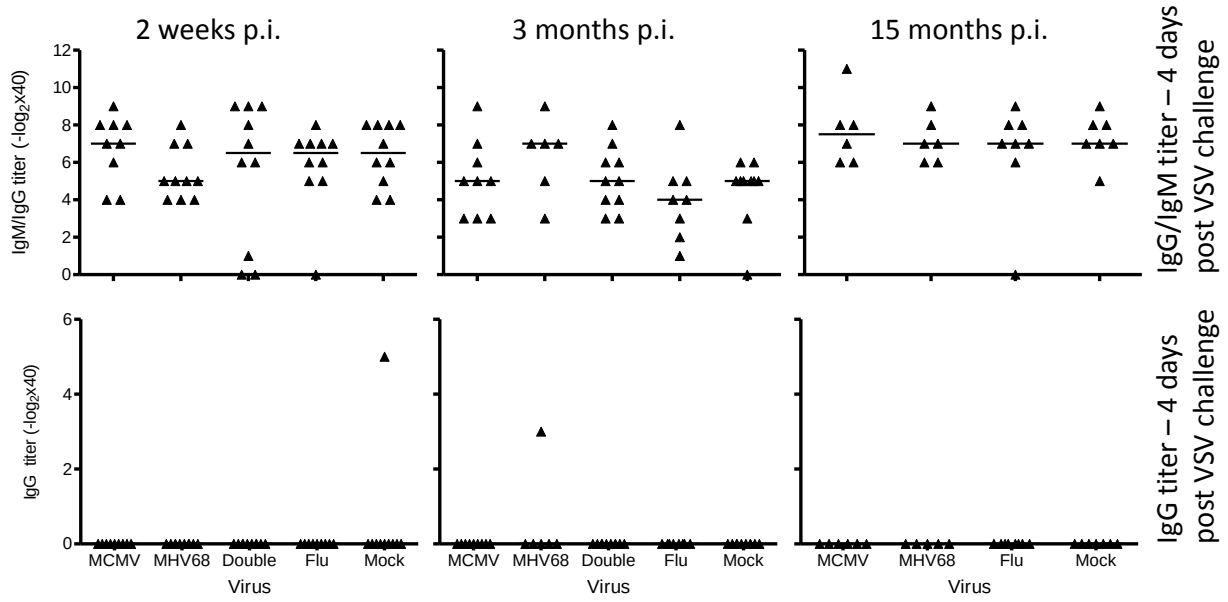

Figure 4.27: No difference in neutralizing immunoglobulin titer against VSV at day 4 post challenge. Serum from the 129B mice primed with MCMV, MHV-68, their combination, flu or mock infected for 2 weeks, 3 months or 15 months at the time of VSV challenge were collected at day 4 post challenge and used in an *in vitro* neutralization assay. Total immunoglobulin titer (upper panels) or the IgG fraction (lower panels) was compared between virus-infected groups and the mock control group. Statistical analysis was performed by the Kruskal-Wallis test followed by Dunns post analysis. Each triangle represents a mouse, horizontal lines are group medians. The data shown here represents pooled data from two independent experiments

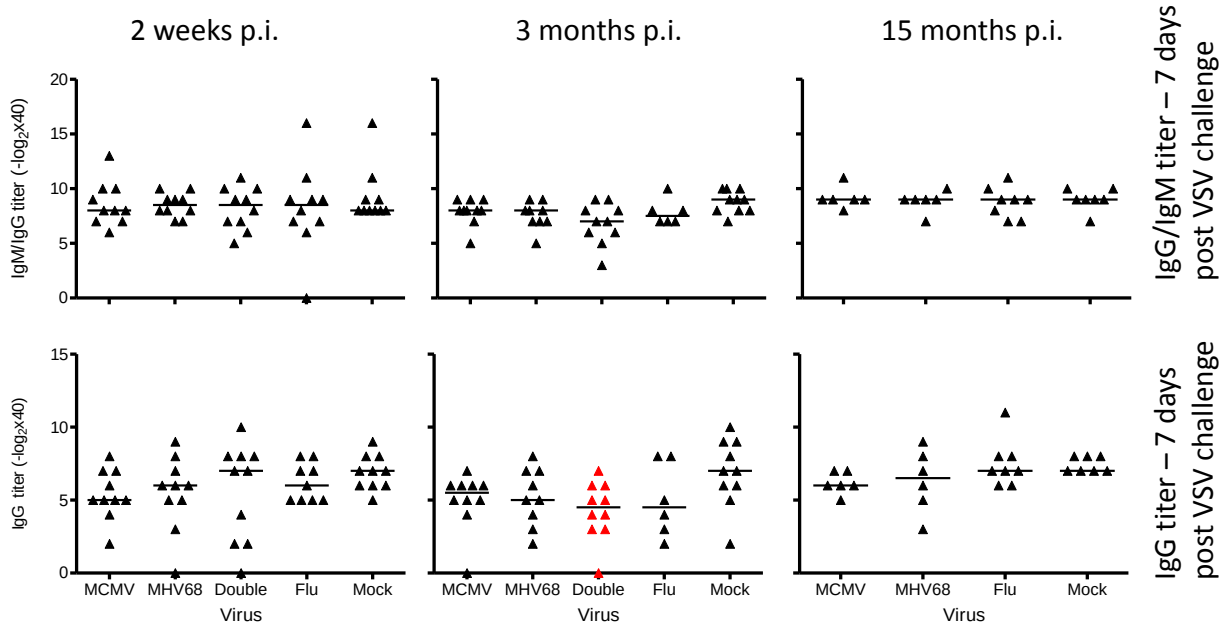

Figure 4.28: Differences in immunoglobulin titer against VSV at 7 days post challenge. Serum from the 129B mice primed with MCMV, MHV-68, their combination, flu or mock infected for 2 weeks, 3 months or 15 months at the time of VSV challenge were collected at day 7 post challenge and used in an *in vitro* neutralization assay. Total immunoglobulin titer (upper panels) or the IgG fraction (lower panels) was compared between virus-infected groups and the mock control group. Statistical analysis was performed by the Kruskal-Wallis test followed by Dunns post analysis. Each triangle represents a mouse, horizontal lines are group medians. The data shown here represents pooled data from two independent experiments

Chapter 5

5.0 Discussion

This work is intended as a comprehensive study of changes in the homeostasis of the adaptive immune system in mice infected with latent herpesviruses. The study provides experimental evidence on the effects of latent herpesvirus infection in mice, and of the challenge with VSV on the adaptive immune system in homeostasis and in response to a new infection. Considering that most people host more than one latent herpesvirus, this study addressed also the question, whether multiple infections with herpesviruses resulted in additive effects that may have affected the immune homeostasis and the adaptive immune response to new infections.

5.1 Effects of persistent herpesvirus infection on CD8 T cells

Herpesviruses impact CD8 T cells by permanently increasing the pool of cells with a memory phenotype. Results from this study showed different effects with α, β, or γ herpesviruses: MCMV, MHV-68 and the triple infection resulted in an incomplete contraction, followed by maintenance of a large EM population after the clearance of the acute phase of infection. On the other hand, the HSV-1 infection resulted in a complete, but transient contraction of the EM pool, down to levels seen upon the non-persistent VACV. This was followed by a second, slowly progressing expansion phase of the EM pool. To my knowledge, no other study has monitored the kinetic of CD8 T cell memory subsets in MHV-68 infected mice. However, others have shown that most of EBV-specific CD8 T cells have a CM phenotype in latently infected individuals, which differs from CMV infection, where the majority of cells bear EM phenotype (139, 140).

Infections with multiple herpesviruses did not result in significant additive effects on the fraction of EM cells in the CD8 pool, as compared to single infection with MCMV or MHV-68. These results could mean that the maximum stable size of the CD8 EM compartment is achieved in a single-infection already, and hence the cells generated against additional herpesviruses cannot add-up to exceed this threshold. The frequency of CD8 T cell response to inflationary MCMV peptides was lower in mice infected with MHV-68 and HSV-1 prior to MCMV infection, as compared to single infection with MCMV. This hypothesis is consistent with the observation that a reduction of HSV-1 specific responses was not seen in the triple infection, where the HSV-1 infection was performed prior to MCMV infection. Alternatively, our results may suggest that the term “memory inflation” as it was defined in experimental MCMV infection (75), is a very limited term that depends on many factors, including the immune status of the host and the history of previous infection with other pathogens. This is supported by the observation that germ-free mice infected with MCMV did not exhibit memory inflation, but that memory inflation could be initiated when the gut flora was replenished in mice carrying latent MCMV (141). Furthermore, another recent study showed that MCMV infected mice, injected with a sub-lethal dose of lipopolysaccharides (LPS) during latency, displayed a transient contraction of the inflationary CD8 T cells, suggesting that pathogen associated molecular patterns or bacterial antigens may also affect the kinetic of memory inflation (142).

MCMV and triple infection resulted in a significant increase in the frequency of CD8 T cells expressing NK markers, an effect that was not as pronounced in groups infected with other herpesviruses. These results suggest that the changes in the phenotype of

CD8 T cells were more prominent in MCMV infection than in infections with other herpesviruses. Therefore, it is likely that the effects seen in the group infected with multiple viruses was mainly due to the MCMV infection.

In both strains of mice used in this study, the frequency of peripheral VSV specific CD8 T cells at day 7 after VSV challenge was lower in mice previously infected with MCMV, consistent with our previously published data (79). This was observed in single infection or in the infection in combination with other herpesviruses, and it occurred regardless of the time-lapse between MCMV infection and VSV challenge. This relative decrease of responding cells was in concert with the absolute increase in the number of CD8 T cells with EM phenotypes and with the increase in the count of total CD8 T cells (Figure 4.12, 4.13, 4.16, and 4.17). Most importantly, there was no difference in the absolute number of the VSV specific CD8 T cells in mice infected with any of the herpesviruses or in the controls. This was observed in all the tested immune compartments, which was consistent with our previous publication, that the count of naïve CD8 cells is not decreased in the blood, spleen or lymph nodes of MCMV-infected mice (79). These results strongly suggest that the absolute number of CD8 T cells generated against new pathogens is relatively similar, regardless of the history of herpesvirus infection. MCMV infection was the only infection that significantly increased the total CD8 T cell count. A recent publication by Mekker *et al.,* showed that MCMV infected mice had increased peripheral CD8 T cell counts and that the effect was more pronounced in the thymectomized mice. Moreover, upon challenge with lymphocytic choriomeningitis virus (LCMV), the absolute number of LCMV specific CD8 T cell from the lungs and spleen of MCMV and mock-infected mice was similar (49). Therefore, previous interpretations of

data from MCMV-infected mice, which showed a decrease in the percentage of CD8 T cells responding to a new infection (79, 143) need to be revised, in order to consider the difference in the total number of CD8 T cells that exists between CMV-infected and non-infected groups.

The expansion of the peripheral EM CD8 T cell pool in mice infected with herpesviruses, and in particular with MCMV, led us to speculate that this might interfere with the activation of new T cells in the draining lymph nodes (79). Guarda *et al.,* have shown that pre-existing EM CD8 T cells (CD62L and CCR7 negative), have the potential to enter draining lymph node through high endothelial venules, mainly due to their expression of CXCR3 during an acute response to *Listeria monocytogenes* challenge (144). A model that considered that the numbers of naïve T cells in CMV infected and uninfected mice are similar, but that the EM CD8 T cells might obstruct the activation of naïve cells in the draining lymph node of CMV infected mice was proposed (Figure 5.1A and 5.1B).

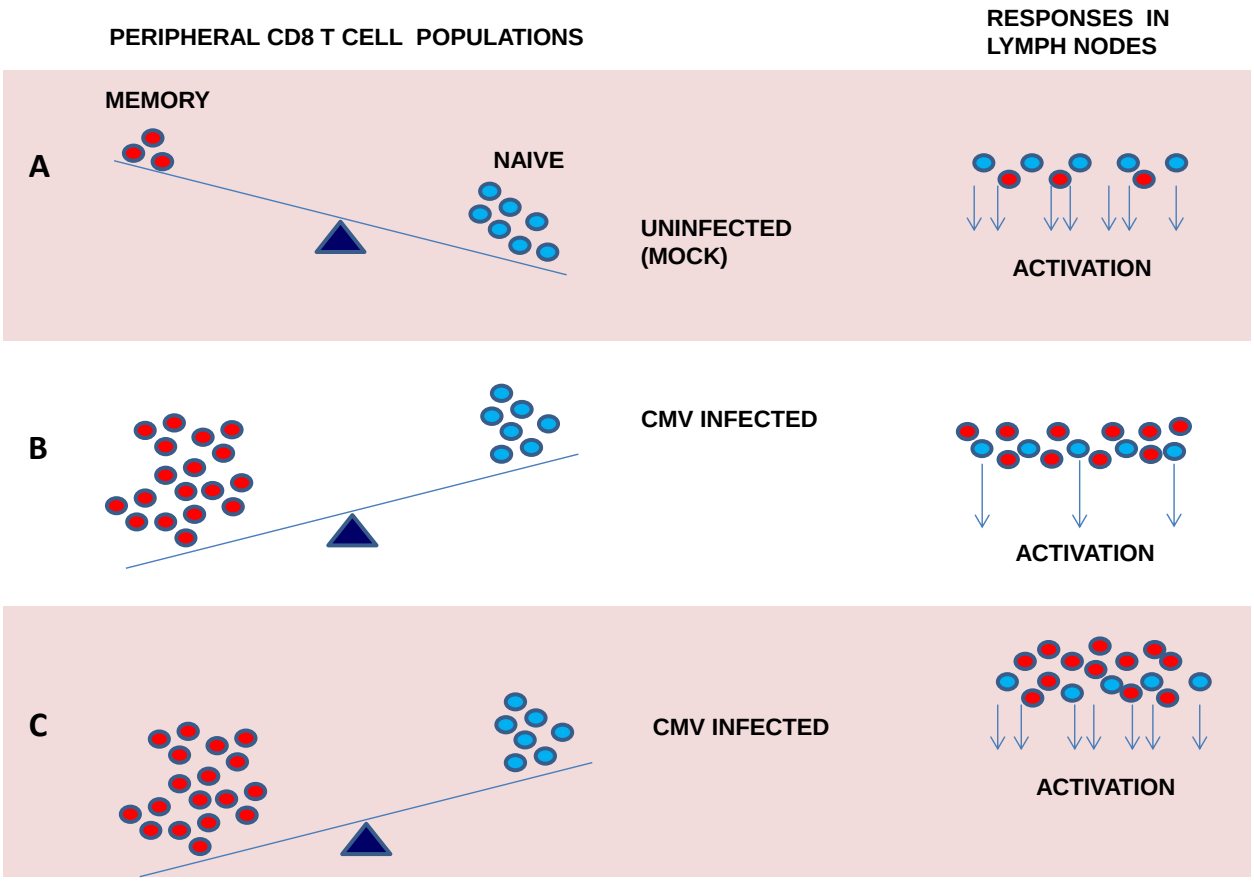

Figure 5.1: Proposed models for CD8 T cells activation in the draining lymph nodes

This hypothesis was tested by analyzing the frequency and absolute numbers of VSV specific CD8 T cells in the draining and non-draining lymphoid organs (spleen and lymph nodes). There was higher number of antigen-specific cells in the draining than in the non-draining lymph nodes suggesting that the activation of CD8 T cells in the draining lymph node was efficient. However, a comparable number of these cells in the lymph nodes of mice infected with herpesviruses and in the control groups was observed, refuting the model from the original hypothesis (Figure 5.1B) and supporting

a model where the increase in the memory cells does not impair the ability of the naïve cell compartment to mount an antigen-specific response (Figure 5.1C). These results were consistent with the observations of Torti *et al.,* who showed that the inflation of CD8 T cells in the peripheral pool of CMV infected mice depends on the continuous activation of CD8 T cells by latently infected non-hematopoietic cells (145). Therefore, their data argued that memory inflation in latent MCMV infection is not a result of T-cell stimulation by professional APCs in the lymph nodes, which would be consistent with our observation that herpesviruses did not impact the activation of VSV-specific CD8 T cells in the lymph nodes.

Our results strongly suggest that infection with a herpesvirus and the consequent memory inflation do not affect the absolute number of VSV specific T cells, despite the homeostatic changes observed in the CD8 T cell compartment. Several groups have proposed to use CMV as a vector to develop T cell-based vaccines (146-149). Importantly, the insertion of additional antigen in CMV-based vaccine vectors does not alter the size of the CMV specific CD8 response, because the EM CD8 T cells specific for the inserted antigen displaces the responses to intrinsic antigens present in the CMV genome (124). We recently published that MCMV infection results in lower frequencies of CTL responses to emerging pathogens (79), thus raising concerns that CMV-based vaccine vectors may have iatrogenic potential, because they may impair the ability of the immune system to protect against environmental pathogens. However, the results shown in this thesis indicate that vaccination with viable CMV vectors would not impair immune system in its ability to respond to emerging infections, and alleviates the concerns about their iatrogenic potential.

5.2 Effects of persistent herpesvirus infection on CD4 T cells

Our results showed that the infection with a single herpesvirus does not have a significant effect on the fraction of EM CD4 T cells unless the mice were infected with a combination of all three herpesviruses used in our study. This suggests that multiple infections with herpesviruses might have an additive effect on CD4 T cell homeostatic changes, in stark contrast with the changes seen in the CD8 T cell compartment.

There was a significantly reduced frequency of regulatory T cells (Tregs) in the MHV-68 group as compared to the control groups and a similar, but non-significant trend in the frequency and absolute numbers was observed in other herpesvirus groups. This may indicate that herpesvirus infection results in a slight reduction in the Tregs populations as an immune strategy to enhance EM CD8 T cell proliferation and pro-inflammatory cytokine production, thus keeping the latent virus in check. This is supported by previous *in vitro* studies that showed that the depletion of Tregs enhances the function and proliferation capacity of virus specific CD8 T cells (150-152), in line with the well-known immunosuppressive function of Tregs (25). Similarly, MCMV infection of IL10 deficient mice showed a reduced latent MCMV genome which suggested an enhanced viral control by the proinflamatory cytokines (133). Future studies of the effect of adoptive transfer of Tregs into mice infected with latent herpesviruses may prove the validity of this hypothesis.

While Th1 CD4 T cells have the potential to control virus replication, there was no significant difference in the fraction or the absolute numbers of VSV specific CD4 T cells responding with Th1 cytokines. This was the case both in the peripheral blood and in

the lymph nodes of mice that have been infected with herpesviruses, implying that Th1 responses against new infections were not affected by the latent herpesviruses.

5.3 Effects of persistent herpesvirus infection on B cells

The fractions of memory B cells were comparable between mice infected for eight months with herpesviruses and control groups. Therefore, our data suggest that the homeostasis of B cells may not be affected by lifelong herpesvirus infection (Figure 4.9). On the other hand, CMV-infected D2B6 mice mounted significantly lower VSV neutralizing titers in the IgG fraction of antibodies at day seven post VSV challenge but not after 14 days, suggesting a delayed Ig class switch (Figure 4.26). Double infected group (MCMV and MHV-68) 129B mice also had low VSV specific IgG titer (Figure 4.28). These data suggest that MCMV infection in isolation or in combination with other herpesviruses may affect primary humoral responses to new infections namely by delaying the Ig class switch. Neutralizing antibody titers produced by plasma cells depends on help from follicular CD4 T cells that facilitate Ig class switch. Recent data from a human study showed that CMV antibody titers correlate directly with the frequency of EM CD4 T cells and impair influenza-specific IgG responses upon vaccination (153). Our data showed that multiple herpesvirus infections have an additive effect on the frequency of EM cells in the CD4 compartment (Figure 4.7), which provided a plausible explanation for the delay of the Ig class-switch. On the other hand, the CD4 T-cell response to VSV was not impaired in herpesvirus infection, arguing that the class-switch delay was caused by changes that were intrinsic to the B cell compartment. Further studies are needed to find out the exact mechanism and consequences of the class switch delay, as well as the exact IgG class that is

predominantly induced upon VSV challenge of latently infected mice. Another interesting question that may be addressed in future studies is whether recall responses would also be delayed.

In general, data from this study indicate that herpesvirus infections impact the T and B cell compartment (Table 5.1), but that the effect in the B cell compartment was mainly on the neutralizing antibody titer, and not on the maintenance of peripheral memory populations. There was a significant reduction of the survival rate in the MCMV infected group; however, this occurred in one experiment only and was not recapitulated in the triple-infection group in the same experiment suggesting this might be a random event not related to CMV infection.

Table 5.1: summarized effects of long life herpesvirus infection on the adaptive immunity

Adaptive immunity	Alpha-herpesvirus (HSV-1)	Beta-herpesvirus (MCMV)	Gamma-herpesvirus (MHV-68)	Combination (triple/double)
Changes in the homeostasis of peripheral CD8 T cells	++	+++	++	+++
Frequency of peripheral CD8 T cells responding to a new infection	+	++	+	++
Absolute number of peripheral CD8 T cells responding to a new infection	-	-	-	-
Total peripheral CD8 T cells	+	+++	+	+++
Homeostasis of peripheral CD4 T cells	+	+	+	++
Peripheral CD4 response to a new infection	-	-	-	-
T cells response in the lymphoid organs	-	-	-	-
Homeostasis of peripheral B cells	-	-	-	-
Antibody response to a new infection	-	++	-	++

+++ *showed a highly significant impact relative to the mock group*

++ *showed significant impact relative to the mock group*

+ *showed borderline significant or a not significant trend relative to the mock group*

- *showed no obvious impact*

Chapter 6

6.0 Conclusions and recommendations for future studies

Data from this study show that herpesvirus infections result in permanent changes of the homeostasis but not of the function of CD4 and CD8 T cells, measured as the ability to respond to a new infection. While previously published data have indicated that the abundance of MCMV-specific CD8 T cells in latently infected mice might impair the response to a neo-antigen in the aged hosts (8, 49, 79), the data presented here show that only the fraction of peripheral VSV specific CD8 T cells but not their absolute number was significantly reduced in mice bearing latent MCMV. Likewise, there was no significant difference in the fraction of lymph node-derived CD8 or CD4 T cells responding to VSV antigen in any mouse group. Therefore, this study argues that herpesvirus infections do not impair the function of the immune system relative to its ability to respond to and protect against an emerging viral challenge. While our data alleviate concerns about the pathogenic potential of natural CMV infection, they also support future prospects of using CMV as a vector to develop T cell-based vaccines. Follow-up studies are needed to clarify if the expansion of EM CD8 T cells caused by CMV infection results in an increase of chronic inflammatory conditions in latently infected hosts.

Finally, this study showed that long-term infection with herpesviruses does not affect the fraction of memory B cells, but the VSV challenge revealed a significant delay in the class switch of VSV-neutralizing antibodies in mice latently infected with MCMV. Future studies should determine whether this delay in the class-switch may also impair the

immune control of emerging pathogens in latently infected hosts, and if the phenomenon is mouse-specific or may be translated to the human situation as well.

References

1. Davison, A. J. 2007. Overview of classification. In *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis*. A. Arvin, G. Campadelli-Fiume, E. Mocarski, P. S. Moore, B. Roizman, R. Whitley, and K. Yamanishi, eds, Cambridge.
2. Whitley, R. J. 1996. Herpesviruses. In *Medical Microbiology*, 4th ed. S. Baron, ed, Galveston (TX).
3. Zhu, F. X., T. Cusano, and Y. Yuan. 1999. Identification of the immediate-early transcripts of Kaposi's sarcoma-associated herpesvirus. *Journal of virology* 73: 5556-5567.
4. Ma, Y., N. Wang, M. Li, S. Gao, L. Wang, Y. Ji, Y. Qi, R. He, Z. Sun, and Q. Ruan. 2011. An antisense transcript in the human cytomegalovirus UL87 gene region. *Virology journal* 8: 515.
5. Noh, C. W., H. J. Cho, H. R. Kang, H. Y. Jin, S. Lee, H. Deng, T. T. Wu, V. Arumugaswami, R. Sun, and M. J. Song. 2012. The virion-associated open reading frame 49 of murine gammaherpesvirus 68 promotes viral replication both in vitro and in vivo as a derepressor of RTA. *Journal of virology* 86: 1109-1118.
6. Keil, G. M., A. Ebeling-Keil, and U. H. Koszinowski. 1984. Temporal regulation of murine cytomegalovirus transcription and mapping of viral RNA synthesized at immediate early times after infection. *Journal of virology* 50: 784-795.
7. Mercadal, S., A. Martinez, B. Nomdedeu, M. Rozman, A. Gaya, O. Salamero, and E. Campo. 2006. Herpes simplex and Epstein-Barr virus lymphadenitis in a patient with chronic lymphocytic leukemia treated with fludarabine. *European journal of haematology* 77: 442-444.
8. Nikolich-Zugich, J. 2008. Ageing and life-long maintenance of T-cell subsets in the face of latent persistent infections. *Nature reviews. Immunology* 8: 512-522.
9. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443: 350-354.
10. Traylen, C. M., H. R. Patel, W. Fondaw, S. Mahatme, J. F. Williams, L. R. Walker, O. F. Dyson, S. Arce, and S. M. Akula. 2011. Virus reactivation: a panoramic view in human infections. *Future virology* 6: 451-463.
11. Janeway, C. A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annual review of immunology* 20: 197-216.
12. Born, W. K., N. Jin, M. K. Aydintug, J. M. Wands, J. D. French, C. L. Roark, and R. L. O'Brien. 2007. gammadelta T lymphocytes-selectable cells within the innate system? *Journal of clinical immunology* 27: 133-144.
13. Godfrey, D. I., H. R. MacDonald, M. Kronenberg, M. J. Smyth, and L. Van Kaer. 2004. NKT cells: what's in a name? *Nature reviews. Immunology* 4: 231-237.
14. Malnati, M. S., M. Marti, T. LaVaute, D. Jaraquemada, W. Biddison, R. DeMars, and E. O. Long. 1992. Processing pathways for presentation of cytosolic antigen to MHC class II-restricted T cells. *Nature* 357: 702-704.

15. Wherry, E. J., and R. Ahmed. 2004. Memory CD8 T-cell differentiation during viral infection. *Journal of virology* 78: 5535-5545.
16. Kunkel, E. J., and E. C. Butcher. 2002. Chemokines and the tissue-specific migration of lymphocytes. *Immunity* 16: 1-4.
17. von Andrian, U. H., and C. R. Mackay. 2000. T-cell function and migration. Two sides of the same coin. *The New England journal of medicine* 343: 1020-1034.
18. Ferguson, A. R., and V. H. Engelhard. 2010. CD8 T cells activated in distinct lymphoid organs differentially express adhesion proteins and coexpress multiple chemokine receptors. *J Immunol* 184: 4079-4086.
19. Dutton, R. W., L. M. Bradley, and S. L. Swain. 1998. T cell memory. *Annual review of immunology* 16: 201-223.
20. Masopust, D., S. M. Kaech, E. J. Wherry, and R. Ahmed. 2004. The role of programming in memory T-cell development. *Current opinion in immunology* 16: 217-225.
21. Luckheeram, R. V., R. Zhou, A. D. Verma, and B. Xia. 2012. CD4(+)T cells: differentiation and functions. *Clinical & developmental immunology* 2012: 925135.
22. Zhu, J., and W. E. Paul. 2010. Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. *Immunological reviews* 238: 247-262.
23. Fazilleau, N., L. Mark, L. J. McHeyzer-Williams, and M. G. McHeyzer-Williams. 2009. Follicular helper T cells: lineage and location. *Immunity* 30: 324-335.
24. King, C., S. G. Tangye, and C. R. Mackay. 2008. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annual review of immunology* 26: 741-766.
25. Shevach, E. M. 2011. Biological functions of regulatory T cells. *Advances in immunology* 112: 137-176.
26. Davis, A. M., K. A. Hagan, L. A. Matthews, G. Bajwa, M. A. Gill, M. Gale, Jr., and J. D. Farrar. 2008. Blockade of virus infection by human CD4+ T cells via a cytokine relay network. *J Immunol* 180: 6923-6932.
27. Whitmire, J. K. 2011. Induction and function of virus-specific CD4+ T cell responses. *Virology* 411: 216-228.
28. Romagnani, S. 1999. Th1/Th2 cells. *Inflammatory bowel diseases* 5: 285-294.
29. Kidd, P. 2003. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Alternative medicine review : a journal of clinical therapeutic* 8: 223-246.
30. Itano, A. A., and M. K. Jenkins. 2003. Antigen presentation to naive CD4 T cells in the lymph node. *Nature immunology* 4: 733-739.
31. Steinman, R. M., M. Pack, and K. Inaba. 1997. Dendritic cells in the T-cell areas of lymphoid organs. *Immunological reviews* 156: 25-37.
32. O'Sullivan, B., and R. Thomas. 2003. CD40 and dendritic cell function. *Critical reviews in immunology* 23: 83-107.
33. Casamayor-Palleja, M., M. Khan, and I. C. MacLennan. 1995. A subset of CD4+ memory T cells contains preformed CD40 ligand that is rapidly but transiently expressed on their surface after activation through the T cell receptor complex. *The Journal of experimental medicine* 181: 1293-1301.

34. Lanzavecchia, A. 1985. Antigen-specific interaction between T and B cells. *Nature* 314: 537-539.
35. Shlomchik, M. J., and F. Weisel. 2012. Germinal center selection and the development of memory B and plasma cells. *Immunological reviews* 247: 52-63.
36. MacLennan, I. C. 1994. Germinal centers. *Annual review of immunology* 12: 117-139.
37. Lazuardi, L., B. Jenewein, A. M. Wolf, G. Pfister, A. Tzankov, and B. Grubeck-Loebenstein. 2005. Age-related loss of naive T cells and dysregulation of T-cell/B-cell interactions in human lymph nodes. *Immunology* 114: 37-43.
38. Hakim, F. T., and R. E. Gress. 2007. Immunosenescence: deficits in adaptive immunity in the elderly. *Tissue antigens* 70: 179-189.
39. Ferrando-Martinez, S., E. Ruiz-Mateos, A. Hernandez, E. Gutierrez, M. Rodriguez-Mendez Mdel, A. Ordonez, and M. Leal. 2011. Age-related deregulation of naive T cell homeostasis in elderly humans. *Age* 33: 197-207.
40. Pinti, M., M. Nasi, E. Lugli, L. Gibellini, L. Bertoncelli, E. Roat, S. De Biasi, C. Mussini, and A. Cossarizza. 2010. T cell homeostasis in centenarians: from the thymus to the periphery. *Current pharmaceutical design* 16: 597-603.
41. Derhovanessian, E., A. Larbi, and G. Pawelec. 2009. Biomarkers of human immunosenescence: impact of Cytomegalovirus infection. *Current opinion in immunology* 21: 440-445.
42. Wikby, A., P. Maxson, J. Olsson, B. Johansson, and F. G. Ferguson. 1998. Changes in CD8 and CD4 lymphocyte subsets, T cell proliferation responses and non-survival in the very old: the Swedish longitudinal OCTO-immune study. *Mechanisms of ageing and development* 102: 187-198.
43. Yager, E. J., M. Ahmed, K. Lanzer, T. D. Randall, D. L. Woodland, and M. A. Blackman. 2008. Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *The Journal of experimental medicine* 205: 711-723.
44. Cicin-Sain, L., S. Smyk-Pearson, N. Currier, L. Byrd, C. Koudelka, T. Robinson, G. Swarbrick, S. Tackitt, A. Legasse, M. Fischer, D. Nikolich-Zugich, B. Park, T. Hobbs, C. J. Doane, M. Mori, M. K. Axthelm, D. A. Lewinsohn, and J. Nikolich-Zugich. 2010. Loss of naive T cells and repertoire constriction predict poor response to vaccination in old primates. *J Immunol* 184: 6739-6745.
45. Swain, S., K. Clise-Dwyer, and L. Haynes. 2005. Homeostasis and the age-associated defect of CD4 T cells. *Seminars in immunology* 17: 370-377.
46. Effros, R. B., and R. L. Walford. 1983. The immune response of aged mice to influenza: diminished T-cell proliferation, interleukin 2 production and cytotoxicity. *Cellular immunology* 81: 298-305.
47. Cancro, M. P., Y. Hao, J. L. Scholz, R. L. Riley, D. Frasca, D. K. Dunn-Walters, and B. B. Blomberg. 2009. B cells and aging: molecules and mechanisms. *Trends in immunology* 30: 313-318.
48. Sauce, D., M. Larsen, S. Fastenackels, A. Duperrier, M. Keller, B. Grubeck-Loebenstein, C. Ferrand, P. Debre, D. Sidi, and V. Appay. 2009. Evidence of premature immune aging in patients thymectomized during early childhood. *The Journal of clinical investigation* 119: 3070-3078.

49. Mekker, A., V. S. Tchang, L. Haeberli, A. Oxenius, A. Trkola, and U. Karrer. 2012. Immune senescence: relative contributions of age and cytomegalovirus infection. *PLoS pathogens* 8: e1002850.
50. Blackman, M. A., and D. L. Woodland. 2011. The narrowing of the CD8 T cell repertoire in old age. *Current opinion in immunology* 23: 537-542.
51. Chayavichitsilp, P., J. V. Buckwalter, A. C. Krakowski, and S. F. Friedlander. 2009. Herpes simplex. *Pediatrics in review / American Academy of Pediatrics* 30: 119-129; quiz 130.
52. Andersson-Ellstrom, A., B. Svennerholm, and L. Forssman. 1995. Prevalence of antibodies to herpes simplex virus types 1 and 2, Epstein-Barr virus and cytomegalovirus in teenage girls. *Scandinavian journal of infectious diseases* 27: 315-318.
53. Rosenthal, S. L., L. R. Stanberry, F. M. Biro, M. Slaoui, M. Francotte, M. Koutsoukos, M. Hayes, and D. I. Bernstein. 1997. Seroprevalence of herpes simplex virus types 1 and 2 and cytomegalovirus in adolescents. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 24: 135-139.
54. Freeman, M. L., B. S. Sheridan, R. H. Bonneau, and R. L. Hendricks. 2007. Psychological stress compromises CD8+ T cell control of latent herpes simplex virus type 1 infections. *J Immunol* 179: 322-328.
55. Padgett, D. A., J. F. Sheridan, J. Dorne, G. G. Berntson, J. Candelora, and R. Glaser. 1998. Social stress and the reactivation of latent herpes simplex virus type 1. *Proceedings of the National Academy of Sciences of the United States of America* 95: 7231-7235.
56. Bonneau, R. H. 1996. Stress-induced effects on integral immune components involved in herpes simplex virus (HSV)-specific memory cytotoxic T lymphocyte activation. *Brain, behavior, and immunity* 10: 139-163.
57. Lang, A., J. D. Brien, and J. Nikolich-Zugich. 2009. Inflation and long-term maintenance of CD8 T cells responding to a latent herpesvirus depend upon establishment of latency and presence of viral antigens. *J Immunol* 183: 8077-8087.
58. Lang, A., J. D. Brien, I. Messaoudi, and J. Nikolich-Zugich. 2008. Age-related dysregulation of CD8+ T cell memory specific for a persistent virus is independent of viral replication. *J Immunol* 180: 4848-4857.
59. Lang, A., and J. Nikolich-Zugich. 2011. Functional CD8 T cell memory responding to persistent latent infection is maintained for life. *J Immunol* 187: 3759-3768.
60. Johnson, A. J., C. F. Chu, and G. N. Milligan. 2008. Effector CD4+ T-cell involvement in clearance of infectious herpes simplex virus type 1 from sensory ganglia and spinal cords. *Journal of virology* 82: 9678-9688.
61. Frank, G. M., A. J. Lepisto, M. L. Freeman, B. S. Sheridan, T. L. Cherpes, and R. L. Hendricks. 2010. Early CD4(+) T cell help prevents partial CD8(+) T cell exhaustion and promotes maintenance of Herpes Simplex Virus 1 latency. *J Immunol* 184: 277-286.
62. Staras, S. A., S. C. Dollard, K. W. Radford, W. D. Flanders, R. F. Pass, and M. J. Cannon. 2006. Seroprevalence of cytomegalovirus infection in the United States,

1988-1994. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 43: 1143-1151.

63. Cannon, M. J., D. S. Schmid, and T. B. Hyde. 2010. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. *Reviews in medical virology* 20: 202-213.
64. Vancikova, Z., and P. Dvorak. 2001. Cytomegalovirus infection in immunocompetent and immunocompromised individuals--a review. *Current drug targets. Immune, endocrine and metabolic disorders* 1: 179-187.
65. Wattre, P., A. Dewilde, and P. E. Lobert. 1995. [Current status of human cytomegalovirus disease]. *La Revue de medecine interne / fondee ... par la Societe nationale francaise de medecine interne* 16: 354-367.
66. Wikby, A., B. Johansson, J. Olsson, S. Lofgren, B. O. Nilsson, and F. Ferguson. 2002. Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. *Experimental gerontology* 37: 445-453.
67. Looney, R. J., A. Falsey, D. Campbell, A. Torres, J. Kolassa, C. Brower, R. McCann, M. Menegus, K. McCormick, M. Frampton, W. Hall, and G. N. Abraham. 1999. Role of cytomegalovirus in the T cell changes seen in elderly individuals. *Clinical immunology* 90: 213-219.
68. Olsson, J., A. Wikby, B. Johansson, S. Lofgren, B. O. Nilsson, and F. G. Ferguson. 2000. Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study. *Mechanisms of ageing and development* 121: 187-201.
69. Pawelec, G., A. Akbar, C. Caruso, R. Solana, B. Grubeck-Loebenstein, and A. Wikby. 2005. Human immunosenescence: is it infectious? *Immunological reviews* 205: 257-268.
70. Akbar, A. N., and J. M. Fletcher. 2005. Memory T cell homeostasis and senescence during aging. *Current opinion in immunology* 17: 480-485.
71. Le Saux, S., C. M. Weyand, and J. J. Goronzy. 2012. Mechanisms of immunosenescence: lessons from models of accelerated immune aging. *Annals of the New York Academy of Sciences* 1247: 69-82.
72. Koch, S., A. Larbi, D. Ozcelik, R. Solana, C. Gouttefangeas, S. Attig, A. Wikby, J. Strindhall, C. Franceschi, and G. Pawelec. 2007. Cytomegalovirus infection: a driving force in human T cell immunosenescence. *Annals of the New York Academy of Sciences* 1114: 23-35.
73. Pawelec, G., Q. Ouyang, G. Colonna-Romano, G. Candore, D. Lio, and C. Caruso. 2002. Is human immunosenescence clinically relevant? Looking for 'immunological risk phenotypes'. *Trends in immunology* 23: 330-332.
74. Munks, M. W., K. S. Cho, A. K. Pinto, S. Sierro, P. Klenerman, and A. B. Hill. 2006. Four distinct patterns of memory CD8 T cell responses to chronic murine cytomegalovirus infection. *J Immunol* 177: 450-458.
75. Karrer, U., S. Sierro, M. Wagner, A. Oxenius, H. Hengel, U. H. Koszinowski, R. E. Phillips, and P. Klenerman. 2003. Memory inflation: continuous accumulation of antiviral CD8+ T cells over time. *J Immunol* 170: 2022-2029.

76. Holtappels, R., M. F. Pahl-Seibert, D. Thomas, and M. J. Reddehase. 2000. Enrichment of immediate-early 1 (m123/pp89) peptide-specific CD8 T cells in a pulmonary CD62L(lo) memory-effector cell pool during latent murine cytomegalovirus infection of the lungs. *Journal of virology* 74: 11495-11503.
77. Snyder, C. M., K. S. Cho, E. L. Bonnett, S. van Dommelen, G. R. Shellam, and A. B. Hill. 2008. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. *Immunity* 29: 650-659.
78. Simon, C. O., R. Holtappels, H. M. Tervo, V. Bohm, T. Daubner, S. A. Oehrlein-Karpi, B. Kuhnapfel, A. Renzaho, D. Strand, J. Podlech, M. J. Reddehase, and N. K. Grzimek. 2006. CD8 T cells control cytomegalovirus latency by epitope-specific sensing of transcriptional reactivation. *Journal of virology* 80: 10436-10456.
79. Cicin-Sain, L., J. D. Brien, J. L. Uhrlaub, A. Drabig, T. F. Marandu, and J. Nikolich-Zugich. 2012. Cytomegalovirus infection impairs immune responses and accentuates T-cell pool changes observed in mice with aging. *PLoS pathogens* 8: e1002849.
80. Widmann, T., U. Sester, B. C. Gartner, J. Schubert, M. Pfreundschuh, H. Kohler, and M. Sester. 2008. Levels of CMV specific CD4 T cells are dynamic and correlate with CMV viremia after allogeneic stem cell transplantation. *PLoS One* 3: e3634.
81. Fletcher, J. M., M. Vukmanovic-Stejic, P. J. Dunne, K. E. Birch, J. E. Cook, S. E. Jackson, M. Salmon, M. H. Rustin, and A. N. Akbar. 2005. Cytomegalovirus-specific CD4+ T cells in healthy carriers are continuously driven to replicative exhaustion. *J Immunol* 175: 8218-8225.
82. Walton, S. M., N. Torti, S. Mandaric, and A. Oxenius. 2011. T-cell help permits memory CD8(+) T-cell inflation during cytomegalovirus latency. *European journal of immunology* 41: 2248-2259.
83. Snyder, C. M., A. Loewendorf, E. L. Bonnett, M. Croft, C. A. Benedict, and A. B. Hill. 2009. CD4+ T cell help has an epitope-dependent impact on CD8+ T cell memory inflation during murine cytomegalovirus infection. *J Immunol* 183: 3932-3941.
84. Walton, S. M., P. Wyrsch, M. W. Munks, A. Zimmermann, H. Hengel, A. B. Hill, and A. Oxenius. 2008. The dynamics of mouse cytomegalovirus-specific CD4 T cell responses during acute and latent infection. *J Immunol* 181: 1128-1134.
85. Trzonkowski, P., J. Mysliwska, E. Szmit, J. Wieckiewicz, K. Lukaszuk, L. B. Brydak, M. Machala, and A. Mysliwski. 2003. Association between cytomegalovirus infection, enhanced proinflammatory response and low level of anti-hemagglutinins during the anti-influenza vaccination--an impact of immunosenescence. *Vaccine* 21: 3826-3836.
86. den Elzen, W. P., A. C. Vossen, H. J. Cools, R. G. Westendorp, A. C. Kroes, and J. Gussekloo. 2011. Cytomegalovirus infection and responsiveness to influenza vaccination in elderly residents of long-term care facilities. *Vaccine* 29: 4869-4874.
87. Polic, B., H. Hengel, A. Krmpotic, J. Trgovcich, I. Pavic, P. Luccaronin, S. Jonjic, and U. H. Koszinowski. 1998. Hierarchical and redundant lymphocyte subset

control precludes cytomegalovirus replication during latent infection. *The Journal of experimental medicine* 188: 1047-1054.

88. Middeldorp, J. M., A. A. Brink, A. J. van den Brule, and C. J. Meijer. 2003. Pathogenic roles for Epstein-Barr virus (EBV) gene products in EBV-associated proliferative disorders. *Critical reviews in oncology/hematology* 45: 1-36.
89. Thompson, M. P., and R. Kurzrock. 2004. Epstein-Barr virus and cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 10: 803-821.
90. Chan, C. W., A. K. Chiang, K. H. Chan, and A. S. Lau. 2003. Epstein-Barr virus-associated infectious mononucleosis in Chinese children. *The Pediatric infectious disease journal* 22: 974-978.
91. Fafi-Kremer, S., P. Morand, J. P. Brion, P. Pavese, M. Baccard, R. Germi, O. Genoulaz, S. Nicod, M. Jolivet, R. W. Ruigrok, J. P. Stahl, and J. M. Seigneurin. 2005. Long-term shedding of infectious epstein-barr virus after infectious mononucleosis. *The Journal of infectious diseases* 191: 985-989.
92. Hislop, A. D., M. E. Ressing, D. van Leeuwen, V. A. Pudney, D. Horst, D. Koppers-Lalic, N. P. Croft, J. J. Neefjes, A. B. Rickinson, and E. J. Wiertz. 2007. A CD8+ T cell immune evasion protein specific to Epstein-Barr virus and its close relatives in Old World primates. *The Journal of experimental medicine* 204: 1863-1873.
93. Delecluse, H. J., R. Feederle, B. O'Sullivan, and P. Taniere. 2007. Epstein Barr virus-associated tumours: an update for the attention of the working pathologist. *Journal of clinical pathology* 60: 1358-1364.
94. Simas, J. P., and S. Efstathiou. 1998. Murine gammaherpesvirus 68: a model for the study of gammaherpesvirus pathogenesis. *Trends in microbiology* 6: 276-282.
95. Efstathiou, S., Y. M. Ho, S. Hall, C. J. Styles, S. D. Scott, and U. A. Gompels. 1990. Murine herpesvirus 68 is genetically related to the gammaherpesviruses Epstein-Barr virus and herpesvirus saimiri. *The Journal of general virology* 71 (Pt 6): 1365-1372.
96. Virgin, H. W. t., P. Latreille, P. Wamsley, K. Hallsworth, K. E. Weck, A. J. Dal Canto, and S. H. Speck. 1997. Complete sequence and genomic analysis of murine gammaherpesvirus 68. *Journal of virology* 71: 5894-5904.
97. Blaskovic, D., M. Stancekova, J. Svobodova, and J. Mistrikova. 1980. Isolation of five strains of herpesviruses from two species of free living small rodents. *Acta virologica* 24: 468.
98. Nash, A. A., B. M. Dutia, J. P. Stewart, and A. J. Davison. 2001. Natural history of murine gamma-herpesvirus infection. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 356: 569-579.
99. Flano, E., S. M. Husain, J. T. Sample, D. L. Woodland, and M. A. Blackman. 2000. Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages. *J Immunol* 165: 1074-1081.
100. Flano, E., I. J. Kim, D. L. Woodland, and M. A. Blackman. 2002. Gamma-herpesvirus latency is preferentially maintained in splenic germinal center and memory B cells. *The Journal of experimental medicine* 196: 1363-1372.

101. Freeman, M. L., K. G. Lanzer, T. Cookenham, B. Peters, J. Sidney, T. T. Wu, R. Sun, D. L. Woodland, A. Sette, and M. A. Blackman. 2010. Two kinetic patterns of epitope-specific CD8 T-cell responses following murine gammaherpesvirus 68 infection. *Journal of virology* 84: 2881-2892.
102. Hislop, A. D., M. Kuo, A. B. Drake-Lee, A. N. Akbar, W. Bergler, N. Hammerschmitt, N. Khan, U. Palendira, A. M. Leese, J. M. Timms, A. I. Bell, C. D. Buckley, and A. B. Rickinson. 2005. Tonsillar homing of Epstein-Barr virus-specific CD8+ T cells and the virus-host balance. *The Journal of clinical investigation* 115: 2546-2555.
103. Sparks-Thissen, R. L., D. C. Braaten, S. Kreher, S. H. Speck, and H. W. t. Virgin. 2004. An optimized CD4 T-cell response can control productive and latent gammaherpesvirus infection. *Journal of virology* 78: 6827-6835.
104. Christensen, J. P., R. D. Cardin, K. C. Branum, and P. C. Doherty. 1999. CD4(+) T cell-mediated control of a gamma-herpesvirus in B cell-deficient mice is mediated by IFN-gamma. *Proceedings of the National Academy of Sciences of the United States of America* 96: 5135-5140.
105. Stuller, K. A., S. S. Cush, and E. Flano. 2010. Persistent gamma-herpesvirus infection induces a CD4 T cell response containing functionally distinct effector populations. *J Immunol* 184: 3850-3856.
106. Alanio, C., I. Bouvier, H. Jusforgues-Saklani, and M. L. Albert. 2013. Tracking antigen-specific CD8(+) T cells using MHC class I multimers. *Methods in molecular biology* 960: 309-326.
107. Casalegno-Garduno, R., A. Schmitt, J. Yao, X. Wang, X. Xu, M. Freund, and M. Schmitt. 2010. Multimer technologies for detection and adoptive transfer of antigen-specific T cells. *Cancer immunology, immunotherapy : CII* 59: 195-202.
108. Kosor, E., A. Gagro, V. Drazenovic, I. Kuzman, T. Jeren, S. Rakusic, S. Rabatic, A. Markotic, K. Gotovac, A. Sabioncello, E. Cecuk, V. Kerhin-Brkljacic, I. Gjenero-Margan, B. Kaic, G. Mlinaric-Galinovic, A. Kastelan, and D. Dekaris. 2003. [MHC tetramers: tracking specific immunity]. *Acta medica Croatica : casopis Hravatske akademije medicinskih znanosti* 57: 255-259.
109. Batard, P., D. A. Peterson, E. Devevre, P. Guillaume, J. C. Cerottini, D. Rimoldi, D. E. Speiser, L. Winther, and P. Romero. 2006. Dextramers: new generation of fluorescent MHC class I/peptide multimers for visualization of antigen-specific CD8+ T cells. *Journal of immunological methods* 310: 136-148.
110. Smithey, M. J., G. Li, V. Venturi, M. P. Davenport, and J. Nikolich-Zugich. 2012. Lifelong Persistent Viral Infection Alters the Naive T Cell Pool, Impairing CD8 T Cell Immunity in Late Life. *J Immunol*.
111. Nugent, C. T., R. M. Wolcott, R. Chervenak, and S. R. Jennings. 1994. Analysis of the cytolytic T-lymphocyte response to herpes simplex virus type 1 glycoprotein B during primary and secondary infection. *Journal of virology* 68: 7644-7648.
112. Mueller, S. N., C. M. Jones, W. Chen, Y. Kawaoka, M. R. Castrucci, W. R. Heath, and F. R. Carbone. 2003. The early expression of glycoprotein B from herpes simplex virus can be detected by antigen-specific CD8+ T cells. *Journal of virology* 77: 2445-2451.

113. Geginat, G., T. Ruppert, H. Hengel, R. Holtappels, and U. H. Koszinowski. 1997. IFN-gamma is a prerequisite for optimal antigen processing of viral peptides in vivo. *J Immunol* 158: 3303-3310.
114. Snyder, C. M., K. S. Cho, E. L. Bonnett, J. E. Allan, and A. B. Hill. 2011. Sustained CD8+ T cell memory inflation after infection with a single-cycle cytomegalovirus. *PLoS pathogens* 7: e1002295.
115. Sakita, I., H. Horig, R. Sun, F. Wang, and S. G. Nathenson. 1996. In vivo CTL immunity can be elicited by in vitro reconstituted MHC/peptide complex. *Journal of immunological methods* 192: 105-115.
116. Jordan, S., J. Krause, A. Prager, M. Mitrovic, S. Jonjic, U. H. Koszinowski, and B. Adler. 2011. Virus progeny of murine cytomegalovirus bacterial artificial chromosome pSM3fr show reduced growth in salivary Glands due to a fixed mutation of MCK-2. *Journal of virology* 85: 10346-10353.
117. Adler, H., M. Messerle, and U. H. Koszinowski. 2001. Virus reconstituted from infectious bacterial artificial chromosome (BAC)-cloned murine gammaherpesvirus 68 acquires wild-type properties in vivo only after excision of BAC vector sequences. *Journal of virology* 75: 5692-5696.
118. Lang, A., and J. Nikolich-Zugich. 2005. Development and migration of protective CD8+ T cells into the nervous system following ocular herpes simplex virus-1 infection. *J Immunol* 174: 2919-2925.
119. Rudd, B. D., V. Venturi, M. J. Smithey, S. S. Way, M. P. Davenport, and J. Nikolich-Zugich. 2010. Diversity of the CD8+ T cell repertoire elicited against an immunodominant epitope does not depend on the context of infection. *J Immunol* 184: 2958-2965.
120. Blaney, J. E., Jr., E. Nobusawa, M. A. Brehm, R. H. Bonneau, L. M. Mylin, T. M. Fu, Y. Kawaoka, and S. S. Tevethia. 1998. Immunization with a single major histocompatibility complex class I-restricted cytotoxic T-lymphocyte recognition epitope of herpes simplex virus type 2 confers protective immunity. *Journal of virology* 72: 9567-9574.
121. Blazejewska, P., L. Koscinski, N. Viegas, D. Anhlan, S. Ludwig, and K. Schughart. 2011. Pathogenicity of different PR8 influenza A virus variants in mice is determined by both viral and host factors. *Virology* 412: 36-45.
122. Stirnweiss, A., A. Ksienzyk, K. Klages, U. Rand, M. Grashoff, H. Hauser, and A. Kroger. 2010. IFN regulatory factor-1 bypasses IFN-mediated antiviral effects through viperin gene induction. *J Immunol* 184: 5179-5185.
123. Van Bleek, G. M., and S. G. Nathenson. 1990. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2Kb molecule. *Nature* 348: 213-216.
124. Dekhtiarenko, I., M. A. Jarvis, Z. Ruzsics, and L. Cicin-Sain. 2013. The context of gene expression defines the immunodominance hierarchy of cytomegalovirus antigens. *J Immunol* 190: 3399-3409.
125. Voehringer, D., C. Blaser, P. Brawand, D. H. Raulet, T. Hanke, and H. Pircher. 2001. Viral infections induce abundant numbers of senescent CD8 T cells. *J Immunol* 167: 4838-4843.

126. Moser, J. M., J. Gibbs, P. E. Jensen, and A. E. Lukacher. 2002. CD94-NKG2A receptors regulate antiviral CD8(+) T cell responses. *Nature immunology* 3: 189-195.
127. Lohwasser, S., A. Kubota, M. Salcedo, R. H. Lian, and F. Takei. 2001. The non-classical MHC class I molecule Qa-1(b) inhibits classical MHC class I-restricted cytotoxicity of cytotoxic T lymphocytes. *International immunology* 13: 321-327.
128. Cush, S. S., and E. Flano. 2011. KLRG1+NKG2A+ CD8 T cells mediate protection and participate in memory responses during gamma-herpesvirus infection. *J Immunol* 186: 4051-4058.
129. Zhou, J., M. Matsuoka, H. Cantor, R. Homer, and R. I. Enelow. 2008. Cutting edge: engagement of NKG2A on CD8+ effector T cells limits immunopathology in influenza pneumonia. *J Immunol* 180: 25-29.
130. Arlettaz, L., J. Villard, C. de Rham, S. Degermann, B. Chapuis, B. Huard, and E. Roosnek. 2004. Activating CD94:NKG2C and inhibitory CD94:NKG2A receptors are expressed by distinct subsets of committed CD8+ TCR alphabeta lymphocytes. *European journal of immunology* 34: 3456-3464.
131. Jabri, B., J. M. Selby, H. Negulescu, L. Lee, A. I. Roberts, A. Beavis, M. Lopez-Botet, E. C. Ebert, and R. J. Winchester. 2002. TCR specificity dictates CD94/NKG2A expression by human CTL. *Immunity* 17: 487-499.
132. Aandahl, E. M., J. Michaelsson, W. J. Moretto, F. M. Hecht, and D. F. Nixon. 2004. Human CD4+ CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *Journal of virology* 78: 2454-2459.
133. Jones, M., K. Ladell, K. K. Wynn, M. A. Stacey, M. F. Quigley, E. Gostick, D. A. Price, and I. R. Humphreys. 2010. IL-10 restricts memory T cell inflation during cytomegalovirus infection. *J Immunol* 185: 3583-3592.
134. Tomayko, M. M., N. C. Steinel, S. M. Anderson, and M. J. Shlomchik. 2010. Cutting edge: Hierarchy of maturity of murine memory B cell subsets. *J Immunol* 185: 7146-7150.
135. Pape, K. A., J. J. Taylor, R. W. Maul, P. J. Gearhart, and M. K. Jenkins. 2011. Different B cell populations mediate early and late memory during an endogenous immune response. *Science* 331: 1203-1207.
136. Dogan, I., B. Bertocci, V. Vilmont, F. Delbos, J. Megret, S. Storck, C. A. Reynaud, and J. C. Weill. 2009. Multiple layers of B cell memory with different effector functions. *Nature immunology* 10: 1292-1299.
137. Good-Jacobson, K. L., and D. M. Tarlinton. 2012. Multiple routes to B-cell memory. *International immunology* 24: 403-408.
138. Bach, P., E. Kamphuis, B. Odermatt, G. Sutter, C. J. Buchholz, and U. Kalinke. 2007. Vesicular stomatitis virus glycoprotein displaying retrovirus-like particles induce a type I IFN receptor-dependent switch to neutralizing IgG antibodies. *J Immunol* 178: 5839-5847.
139. Hislop, A. D., N. H. Gudgeon, M. F. Callan, C. Fazou, H. Hasegawa, M. Salmon, and A. B. Rickinson. 2001. EBV-specific CD8+ T cell memory: relationships between epitope specificity, cell phenotype, and immediate effector function. *J Immunol* 167: 2019-2029.

140. Torti, N., and A. Oxenius. 2012. T cell memory in the context of persistent herpes viral infections. *Viruses* 4: 1116-1143.
141. Tanaka, K., S. Sawamura, T. Satoh, K. Kobayashi, and S. Noda. 2007. Role of the indigenous microbiota in maintaining the virus-specific CD8 memory T cells in the lung of mice infected with murine cytomegalovirus. *J Immunol* 178: 5209-5216.
142. Campbell, J., J. Trgovcich, M. Kincaid, P. D. Zimmerman, P. Klenerman, S. Sims, and C. H. Cook. 2012. Transient CD8-memory contraction: a potential contributor to latent cytomegalovirus reactivation. *Journal of leukocyte biology* 92: 933-937.
143. Beswick, M., A. Pachnio, S. N. Lauder, C. Sweet, and P. A. Moss. 2013. Antiviral therapy can reverse the development of immune senescence in elderly mice with latent cytomegalovirus infection. *Journal of virology* 87: 779-789.
144. Guarda, G., M. Hons, S. F. Soriano, A. Y. Huang, R. Polley, A. Martin-Fontecha, J. V. Stein, R. N. Germain, A. Lanzavecchia, and F. Sallusto. 2007. L-selectin-negative CCR7- effector and memory CD8+ T cells enter reactive lymph nodes and kill dendritic cells. *Nature immunology* 8: 743-752.
145. Torti, N., S. M. Walton, T. Brocker, T. Rulicke, and A. Oxenius. 2011. Non-hematopoietic cells in lymph nodes drive memory CD8 T cell inflation during murine cytomegalovirus infection. *PLoS pathogens* 7: e1002313.
146. Hansen, S. G., J. C. Ford, M. S. Lewis, A. B. Ventura, C. M. Hughes, L. Coyne-Johnson, N. Whizin, K. Oswald, R. Shoemaker, T. Swanson, A. W. Legasse, M. J. Chiuchiolo, C. L. Parks, M. K. Axthelm, J. A. Nelson, M. A. Jarvis, M. Piatak, Jr., J. D. Lifson, and L. J. Picker. 2011. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. *Nature* 473: 523-527.
147. Tsuda, Y., P. Caposio, C. J. Parkins, S. Botto, I. Messaoudi, L. Cicin-Sain, H. Feldmann, and M. A. Jarvis. 2011. A replicating cytomegalovirus-based vaccine encoding a single Ebola virus nucleoprotein CTL epitope confers protection against Ebola virus. *PLoS neglected tropical diseases* 5: e1275.
148. Klyushnenkova, E. N., D. V. Kouiavskaia, C. J. Parkins, P. Caposio, S. Botto, R. B. Alexander, and M. A. Jarvis. 2012. A cytomegalovirus-based vaccine expressing a single tumor-specific CD8+ T-cell epitope delays tumor growth in a murine model of prostate cancer. *Journal of immunotherapy* 35: 390-399.
149. Slavuljica, I., A. Busche, M. Babic, M. Mitrovic, I. Gasparovic, D. Cekinovic, E. Markova Car, E. Pernjak Pugel, A. Cikovic, V. J. Lisnic, W. J. Britt, U. Koszinowski, M. Messerle, A. Krmpotic, and S. Jonjic. 2010. Recombinant mouse cytomegalovirus expressing a ligand for the NKG2D receptor is attenuated and has improved vaccine properties. *The Journal of clinical investigation* 120: 4532-4545.
150. Li, Y. N., X. L. Liu, F. Huang, H. Zhou, Y. J. Huang, and F. Fang. 2010. CD4+CD25+ regulatory T cells suppress the immune responses of mouse embryo fibroblasts to murine cytomegalovirus infection. *Immunology letters* 131: 131-138.
151. Rushbrook, S. M., S. M. Ward, E. Unitt, S. L. Vowler, M. Lucas, P. Klenerman, and G. J. Alexander. 2005. Regulatory T cells suppress in vitro proliferation of

virus-specific CD8+ T cells during persistent hepatitis C virus infection. *Journal of virology* 79: 7852-7859.

152. Fogg, M., J. R. Murphy, J. Lorch, M. Posner, and F. Wang. 2013. Therapeutic targeting of regulatory T cells enhances tumor-specific CD8+ T cell responses in Epstein-Barr virus associated nasopharyngeal carcinoma. *Virology*.
153. Alonso Arias, R., M. A. Moro-Garcia, A. Echeverria, J. J. Solano-Jaurrieta, F. M. Suarez-Garcia, and C. Lopez-Larrea. 2013. Intensity of the humoral response to cytomegalovirus is associated with the phenotypic and functional status of the immune system. *Journal of virology* 87: 4486-4495.